Hematopoietic Stem Cell Behavior During Cytokine Challenge And Ageing

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Table of Contents

ABBREVIATIONS 3

SUMMARY 6

ZUSAMMENFASSUNG 9

INTRODUCTION 12

Hematopoietic system hierarchy 12

Bone marrow microenvironment 14

Hematopoietic stem cell heterogeneity 15

Proliferative rate of hematopoietic stem and progenitor cells 16

Hematopoietic cell response to stress 19

Ageing of the hematopoietic system 21

Hematopoietic stem cells ageing 22

Potential mechanisms of hematopoietic stem cell ageing 24

Rejuvenation of hematopoietic stem cells 25

Ageing associated inflammation “inflammageing” 25

STUDY AIMS 27

RESEARCH ARTICLE 1 29

RESEARCH ARTICLE 2 49

CONTRIBUTIONS 69

DISCUSSION AND OUTLOOK 73

CONCLUSIONS 78

ACKNOWLEDGEMENTS 79

REFERENCE LIST 82

ABBREVIATIONS

5FU	5 Fluorouracil
AGM	Aorta gonad mesonephros
Akt/PKB	Ak-mouse strain transforming viral proto-oncogene1, protein kinase B
AML	Acute myeloid leukemia
Ang1	Angiopoietin 1
BM	Bone marrow
CAR cells	CXCL12 abundant reticular cells
CD	Cluster of differentiation
Cdk	Cyclin D kinase
Cdc42	Cell division cycle 42
CFSE	5-(and-6)-Carboxyfluorescein Diacetate, Succinimidyl Ester
CFU-E	Colony forming unit – erythrocyte
CFU-G	Colony forming unit – granulocyte
CFU-GM	Colony forming unit – granulocyte macrophage
CFU-GEMM	Colony forming unit – granulocyte erythrocyte macrophage megakaryocyte
CFU-Mk	Colony forming unit – megakaryocyte
CFU-M	Colony forming unit – macrophage
CKI	Cyclin kinase inhibitors
c-Kit	c-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
CLP	Common lymphoid progenitor cells
cMpl	Myeloproliferative leukemia oncogene
CXCL12	C-X-C motif chemokine 12 (SDF1)
CXCR4	C-X-C chemokine receptor type 4
CY	Cyclophosphomide
DNA	Desoxyribonucleic acid
EC	Endothelial cells
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EPCR	Endothelial protein C receptor
Flt3(L)	FMS-like tyrosine kinase (ligand)

G0	Gap 0 cell cycle phase
G1	Gap 1 cell cycle phase
G-CSF	Granulocyte colony stimulating factor
GMP	Granulocyte macrophage progenitor cells
GM-CSF	Granulocyte macrophage colony stimulating factor
HSC	Hematopoietic Stem Cells
HSCT	Hematopoietic stem cell transplantation
HPC	Hematopoietic Progenitor Cells
IFNα/γ	Interferon alpha/gamma
IGF-1	Insulin-like growth factor 1
IL-1α/β	Interleukin 1 alpha/beta
IL-1ra	Interleukin 1 receptor antagonist
JAK2-V617F	Janus kinase 2 mutation in Valine617Phenylalanin
KI	Knock in
KO	Knock out
Lin^-	Lineage negative
LKS	$Lin^-cKit^+Sca1^+$
LPS	Lypopolysaccharide
LyHSC	Lymphoid biased HSCs
M-CSF	Macrophage colony stimulating factor
MDS	Myelodysplastic syndrome
MEP	Megakaryocyte erythrocyte progenitor cells
MIP-2α	Macrophage inflammatory protein 2-alpha
MSC	Mesenchymal stem/stroma cells
MyHSC	Myeloid biased HSC
Mφ	Macrophages
mTOR	Mammalian target of rapamycin
NK cells	Natural Killer cells
PB	Peripheral blood
PBS	Phosphate buffered saline
PDGFRα	Platelet-derived growth factor receptor, alpha
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase,

RANTES	Regulated on activation, normal T cell expressed and secreted
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Sca1	Ly6a lymphocyte antigen 6 complex
SCF	Stem cell factor
S/G2/M	Synthesis/Gap2/Mitosis cell cycle phase
SLAM	Signaling lymphocyte activation molecule
SLAM HSC	LKS $CD48^-$ $CD150^+$
Tie2	TEK endothelial tyrosine kinase
TGFbeta	tissue growth factor beta
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TPO	Thrombopoietin
vWF	von Willebrand factor
Wnt	Wingless oncogene analog
WT	Wild type

SUMMARY

Hematopoiesis is a highly proliferative organ system with approximately $490x10^9$ cells being produced on a daily basis. All mature hematopoietic cells originate from hematopoietic stem cells (HSCs) via developmental intermediates (hematopoietic progenitor cells, HPCs). However, at any given time in steady-state only a minority of HSCs are actively cycling and contributing to blood formation, while the majority of HSCs are quiescent. This changes upon demand, e.g. upon blood loss or infection, where quiescent HSCs can be activated to produce necessary blood cell progeny, a process that involves increased levels of hematopoietic growth factors.

Hematopoietic acting factors are used in clinical medicine to infer with hematopoiesis or compensate for hematopoietic cell deficiencies: the thrombopoietin mimetic or thrombopoietin receptor (cMpl) agonist (Nplate, Romiplostim) is utilized in patients with immune thrombocytopenia to reinforce platelet production; granulocyte colony stimulating factor (G-CSF, Filgrastim) is applied to patients to compensate neutropenias, e.g. after myeloablative treatment or as mobilizing agent for HSCs and HPCs; similarly, the C-X-C chemokine receptor type 4 (CXCR4) antagonist Plerixafor is used to induce HSC and HPC mobilization to peripheral blood prior to HSC/HPC harvest and autologous or allogeneic HSC transplantation. FMS-like tyrosin kinase 3 ligand (Flt3L) is used to mobilize hematopoietic and dendritic cell precursors in clinical studies.

In one part of my thesis work, I addressed the question how these clinically utilized factors affect HSC function. This study demonstrated that enhanced in vivo stimulation with cMpl agonist expands HSCs by driving them into proliferation and maintaining their self-renewal capacity. In contrast, enhanced stimulation with G-CSF had little effect on dormant HSCs, and proliferative phenotypic HSCs did not maintain self-renewal potential. Administration of Flt3L and a CXCR4 antagonist Plerixafor, did not activate dormant HSCs to proliferation, but Flt3L increased the proportion of highly proliferative $Flt3^+$ HPCs. In addition I demonstrated that long-term treatment (over 6 month) with a cMpl agonist lead to increase in numbers of early hematopoietic cell populations of bone marrow, to increased myeloid lineage output, and to increase in CXCL12 abundant reticular cells (CAR), a for hematopoiesis important stroma cell population in bone marrow. Altogether these results suggest that cMpl agonists can be used to expand HSC in vivo. Moreover, cMpl agonists

might be utilized in clinical settings to sensitize normal or malignant cells to cell-cycle phase dependent cytotoxic agents.

In a second, connected part of my studies, I investigated ageing of HSCs. Ageing has been shown to profoundly influence HSC function in mice and humans: aged HSCs were demonstrated to have decreased self-renewal, myelopoiesis-dominant differentiation, and inefficient BM–homing ability. In aged mice, the subpopulation of myeloid-biased HSCs dominates the entire HSC pool. These characteristics of HSC ageing can be experimentally recapitulated by increasing HSC turnover either with multiple injections of myeloablative regimens, such as 5-fluorouracil (5-FU) into young animals, or with several rounds of transplantation (serial transplantation) of young HSC into lethally irradiated animals (‘experimental ageing’). This indicates that hematopoietic ageing is in part featured by accumulation of proliferative history of HSCs, which is regulated by cell-intrinsic and -extrinsic factors.

It was shown by our group and others, that at any given time the HSC pool consists of quiescent (dormant) and actively cycling fractions, with both comparable long-term self-renewing capacities. These fractions do not exist with fixed but rather fluctuating cycling activity over time, resulting in similar turnover of the entire HSC pool at the end of life. Yet, it remains unclear how intrinsic and extrinsic factors influence self-renewal, differentiation, and apoptosis of HSCs during ageing, how these factors contribute to HSC cell fate decisions, what the underlying molecular mechanisms and signature are, and whether the ageing phenotype can be reversed or rejuvenated as a measure for regenerative medicine.

In this part of my thesis work, I demonstrated that aged HSCs with extensive proliferative history increase their quiescence. These quiescent HSCs upon subsequent activation show myeloid biased repopulation. Moreover, aged HSCs can switch from myelopoiesis to lymphopoiesis, when they get activated upon cell cycle induction in a young environment. I also found that the aged bone marrow microenvironment is composed of a different cytokine milieu, with increased levels of inflammatory cytokines, such as IL-1a and IL-1b. These cytokines provide increased differentiation signals to young HSCs as well as aged HSCs, but aged HSCs showed a delayed response. In addition, myeloid biased blood production in an aged context is diminished in absence of IL-1 signaling in respective cytokine KO mice.

Together, my studies revealed how HSC cycling and mature blood cell production by HSCs is modulated by growth factors in various contexts such as inflammation and ageing. These findings might add to novel clinical approaches in settings of hematopoietic deficiencies, ageing, and possibly hematopoietic malignancies.

ZUSAMMENFASSUNG

Die Hämatopoese ist ein hoch proliferatives Organsystem, das täglich ca. $490x10^9$ Zellen produziert. Alle reifen hämatopoetischen Zellen entstehen über Entwicklungszwischenprodukte (hämatopoetischen Vorläuferzellen, HPC) aus hämatopoetischen Stammzellen (HSC). Dennoch sind zu einem bestimmten Zeitpunkt nur eine Minderheit der HSCs aktiv proliferierend und tragen zur Blutbildung bei, während die Mehrheit der HSCs ruhend sind. Dies ändert sich bei Bedarf. Zum Beispiel können nach Blutverlust oder bei Infektion ruhende HSCs aktiviert werden, um den erhöhten Bedarf an Blutzellnachkommen zu produzieren. Dieser Prozess wird durch hämatopoetische Wachstumsfaktoren reguliert.

Hämatopoetisch wirkende Faktoren werden auch in der klinischen Medizin eingesetzt: das Thrombopoietin-Mimetikum bzw. der Thrombopoietin-Rezeptor-Agonist Nplate® (Romiplostim) wird bei Patienten mit Immun-Thrombozytopenie (ITP) verwendet, um die Thrombozytenproduktion zu verstärken; der Granulozyten-Kolonie-stimulierende Faktor (G-CSF, Filgrastim) wird bei Patienten angewendet, um Neutropenie nach Chemotherapie zu reduzieren bzw. um HSCs und HPC in das Blut zur Sammlung vor Blutstammzelltranplantation zu mobilisieren,; ebenso wird der CXC-Chemokin-Rezeptor Typ 4 (CXCR4) -Antagonist Plerixafor verwendet, um HSC und HPC zu mobilisieren; der FMS-like Tyrosinkinase-3-Ligand (Flt3L) wird verwendet, um hämatopoetische und Vorläufer dendritischer Zellen in klinischen Studien zu mobilisieren.

In einem Teil meiner Dissertation erforschte ich die Frage, wie diese klinisch genutzt Faktoren die HSC-Funktion beeinflussen. Ich konnte zeigen, dass eine verstärkte in vivo cMpl-Stimulation die HSC Proliferation unter Aufrechterhaltung des Selbsterneuerungspotentials fördert. Im Gegensatz dazu hatte die Stimulation mit G-CSF eine geringe Wirkung auf ruhende HSCs. Ebenso wurde durch die Verabreichung von Flt3L und CXCR4-Antagonisten keine HSC Proliferation induziert, es kam durch Flt3L jedoch zu einer Proliferation von $Flt3^+$ hämatopoetischen Vorläuferzellen. Darüber hinaus zeigte ich, dass eine Langzeitbehandlung mit einem cMpl-Agonisten (über 6 Monate) zu einer erhöhten Anzahl an frühen hämatopoetischen Zellpopulationen des Knochenmarks, zu einer erhöhten Produktion myeloider Zellen und CXCL12 produzierenden Retikulumzellen (CAR), eine für die Blutbildung wichtige Zellpopulation des Knochenmarkstromas, führt,. Insgesamt legen diese

Ergebnisse nahe, dass cMpl-Agonisten verwendet werden können, um HSC in vivo zu expandieren. Es kann darüber hinaus spekuliert werden, dass cMpl-Agonisten verwendet werden können, um normale oder maligne Zellen der frühen Hämatopoiese gegenüber Zellzyklus abhängigen zytotoxischen Medikamenten zu sensibilisieren.

In einem zweiten, verbundenen Teil meiner Dissertationuntersuchte ich die Alterung der HSCs. Es hat sich gezeigt, dass Alterung die HSC-Funktion bei Mäusen und Menschen entscheidend beeinflusst: alte HSCs haben ein geringeres Selbsterneuerungspotential, differenzieren dominant zu myeloischen Zellen und zeigen ein vermindertes Potential nach Transfer in das Knochenmark zu gehen (verringertes Homing). In älteren Mäusen ist die Subpopulation vorwiegend myeloisch differenzierender HSCs dominannt. Die Charakteristika der HSC Alterung können experimentell durch Erhöhung des HSC Umsatzes/Proliferation entweder durch Injektionen myeloablativer Substanzen wie 5-Fluorouracil (5-FU) in jungen Tieren oder durch serielle HSC Transplantation in letal bestrahlte Tiere rekapituliert werden (experimentelles Altern). Dies zeigt, dass hämatopoetische Alterung zum Teil von extrinsischen Faktoren, zum Teil aber auch durch die Proliferationshistorie HSCs reguliert wird..

Unsere Gruppe und Adere konnten zeigen, dass zu jedem gegebenen Zeitpunkt die HSC Fraktion aus ruhenden (inkativ) und aktiv proliferierenden Pools mit vergleichbarer Langzeit-Erneuerungskapazität besteht. Beide Fraktionen zeigen eine veränderliche Teilungsaktivität, so dass über das Gesamte Leben ein ähnlicher Umsatz des gesamten HSC-Pools erreicht wird. Es ist jedoch unklar, wie intrinsische und extrinsische Faktoren und welche molekularen Mechanismen die Selbsterneuerung, Differenzierung und Apoptose von HSCs während der Alterung bestimmen. Ebenso ist unklar, ob der Alterungs-Phänotyp von HSCs reversibel ist und dies eventl. im Rahmen von therapeutischen Massnahmen der regenerativen Medizin erreicht werden kann.

In diesem Teil meiner Doktorarbeit habe ich gezeigt, dass gealterte HSCs mit umfangreicher proliferativer Historie sich vermehrt im Ruhezustand befinden. Diese ruhenden HSCs zeigen bei forcierter Aktivität eine vermehrt myeloische Differenzierung. Ich konnte ferner feststellenm, dass die gealterte HSCs Tendenz zur Myelopoese auch wieder vermehrt Lymphopoese generieren, wenn sie bei der Zellzyklus-Induktion in einer jungen Umgebung aktiviert zu werden. Ich konnte zeigen, daß die gealterte Knochenmarkmikroumgebung eines verändertes Zytokinmilieu hat. Es finden sich erhöhte

Spiegel von inflammatorischen Zytokinen,wie z.B. IL-1α und IL-1β. Diese Zytokine induzieren Proliferation und eine erhöhte myeloide Differenzierung, wobei die Sensitiviät von jungen HSC höher ist und die gealterten HSC eine verzögerte Reaktion zeigen. In weiteren Untersuchungen konnte ich zeigen, dass in Abwesenheit von IL-1 (knock-out Mäuse) der myeloische Phänotyp von gealterten HSC in alten Mäusen geringer ausgeprägt ist.

Zusammen ergaben meine Untersuchungen, wie Proliferation von HSC und Produktion von reifen Blutzellproduktion von HSCs durch Wachstumsfaktoren in verschiedenen Kontexten wie Entzündungen und Alterung moduliert wird. Diese Ergebnisse könnten letzlich zu neuen klinischen Ansätzen in der Behandlung von hämatopoetischen Defiziten, der hämatopoietischen Alterung und möglicherweise auch hämatopoetischen Neoplasien führen.

INTRODUCTION

Hematopoietic system hierarchy

Hematopoiesis is an active continuous process of production and consumption of mature blood cells throughout the lifetime of an individual. Hematopoiesis is maintained by a rare population of HSCs that are characterized by two basic properties: self-renewal and the ability to produce all types of blood cells including red blood cells, platelets, leukocytes of myeloid lineage, lymphoid lineage, and dendritic cell populations throughout life.

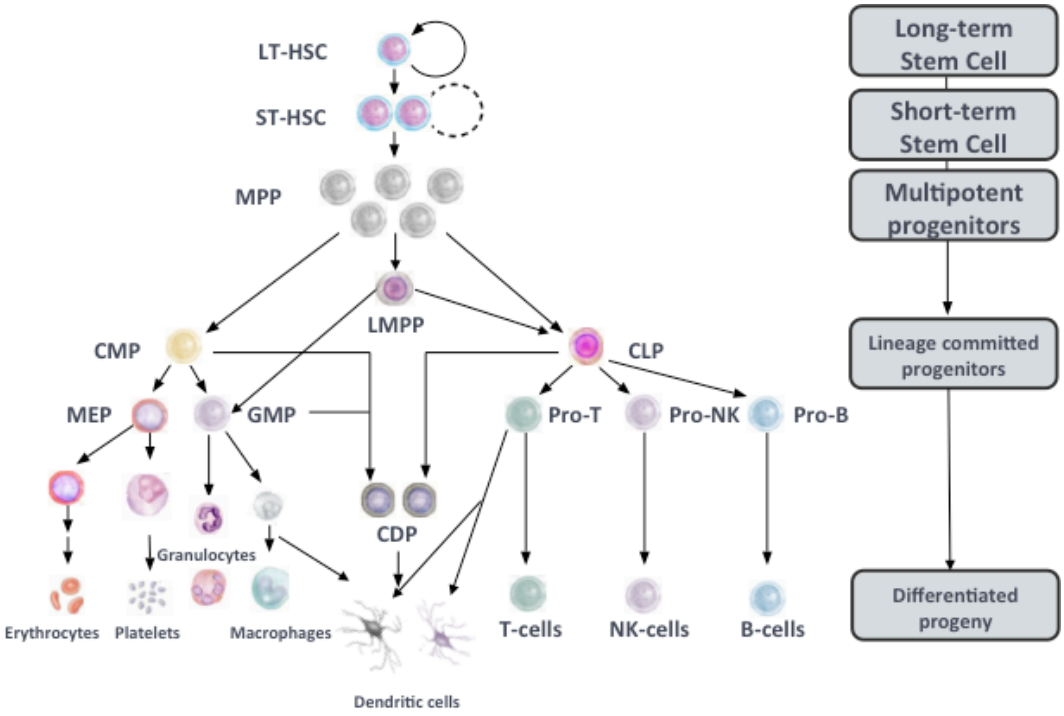


Figure 1. Hierarchy within hematopoietic system. The "tree" like organization of hematopoietic system, where at the apex reside HSCs with long-term self-renewal capacity, that, through multipotent and lineage committed progenitors, generate the differentiated mature cells, with no or limited self-renewal capacity. (Based on Kondo Cell 1997, Akashi Nature 2000, Traver Science 2000, Manz Blood 2001, Manz PNAS 2002, Chicha JEM 2004, Onai Nature Immunology 2007)

Mouse primitive HSCs appear in the yolk sac at embryonic day 7.5 (E7.5) and do not have full adult HSC functionality and mostly form primitive erythroid colonies. HSCs with fully functional properties (long-term, multilineage and substantial repopulation) that are capable of reconstitution myelo-ablated animals develop at E10.5 in the aorta-gonad-mesonephros region (AGM) (Müller et al. 1994; Medvinsky & Dzierzak 1996; de Bruijn et al. 2000). These fully functional HSCs are then found in the placenta and in the fetal liver. The

fetal liver does not generate de novo HSCs, rather it is colonized by HSCs generated at the AGM region, placenta and yolk sac (Dzierzak & Speck 2008). Fetal liver HSCs colonize the thymus and spleen from E11-E12.5. In the post-natal period HSCs migrate to bone marrow (BM) where they reside during adult hematopoiesis. During adulthood, few HSCs are also found in blood circulation, as proven in experiments with parabiotic animals that share a blood circulation, or through repetitive intravenous transplantation of HSCs. Indeed, approximately 1-5% of HSCs enter blood circulation every day and likely a majority of these re-enter the BM again (Gibney et al. 2012; Bhattacharya et al. 2009; Massberg et al. 2007; Wright, Wagers, et al. 2001).

The hematopoietic system is hierarchically organized: HSCs with lifelong self-renewal capacity give rise to multipotent and oligopotent progenitor cells with limited short-term self-renewal capacity. They in turn give rise to precursor cells and mature blood cells with no self-renewal capacity, except for specific subtypes of B and T cells, tissue resident Macrophages (Mφ) and some dendritic cell subsets (Manz & Boettcher 2014) (Figure 1).

Hematopoiesis is controlled in a cell-intrinsic (epigenetic and transcription programs) and in a cell-extrinsic (soluble growth factors, cytokines, hormones, supporting cell types and structures in bone marrow) fashion. Loss of function experiments demonstrated that mice deficient in growth factors develop hematopoietic stem cell dysfunction or hematopoietic lineage deficiencies (stem cell factor (SCF) (McCarthy et al. 1977), thrombopoietin (TPO) (Murone et al. 1998), FMS-like tyrosin kinase 3 (Flt3) (McKenna et al. 2000), Tie2/ Angiopoietin (Ang-1) (Arai et al. 2004), macrophage colony stimulating factor (M-CSF, CSF-1) (Wiktor-Jedrzejczak 1993), granulocyte macrophage colony stimulating factor (GM-CSF, CSF-2) (Stanley et al. 1994), double knock out of CSF-1/CSF-2 (Lieschke, Stanley, et al. 1994), granulocyte colony stimulating factor (G-CSF, CSF-3) (Lieschke, Grail, et al. 1994)). In contrast, gain of function experiments, such as mutations leading to constitutive activation of the downstream growth factor signaling, lead to development of hematopoietic neoplasms. For example JAK2-V617F mutated HSCs can initiate myeloproliferative neoplasm at the single cell level (Lundberg et al. 2014). This indicates that normal hematopoiesis has to be tightly controlled in a cell intrinsic and extrinsic manner, and lack of control might lead to aplasia or neoplasia.

Bone marrow microenvironment

HSCs are maintained in a specialized microenvironment in the BM, called the HSC niche. The HSC niche was conceptually thought to protect HSCs from naturally existing cytotoxic and genotoxic metabolites (Eliasson & Jönsson 2010) and provide HSCs with essential and sufficient factors for their maintenance, self-renewal and differentiation. Recent studies, however, indicate that HSCs themselves can actively respond to stress and activate an unfolded protein response (van Galen et al. 2014).

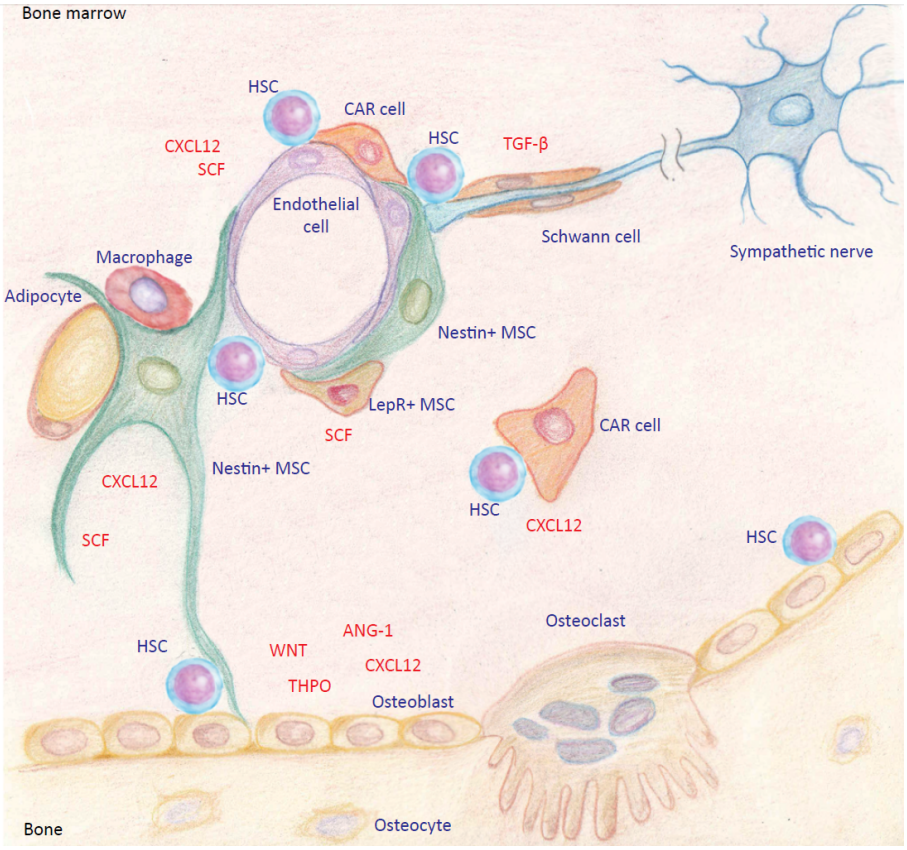


Figure 2. Conceptual illustration of the Bone Marrow Microenvironment. Niche cells (blue text) and niche factors (red text) previously reported are depicted. HSC, hematopoietic Stem Cell; CAR cell, CXCL12 abundant reticular cell; MSC, mesenchymal stromal cell; LepR, Leptin receptor; ANG-1, Angiopoietin-1; SCF, Stem cell factor; THPO, Thrombopoietin; TGF-β, Tumor growth factor-β;WNT, wingless type member.

Niche cells (Figure 2) consist of non-hematopoietic cells (e.g. osteoblasts (Zhang et al. 2003; Calvi et al. 2003; Mercier et al. 2012), endothelial cells (ECs) (Mansour et al. 2012), adipocytes (Naveiras et al. 2009), Schwann cells (Yamazaki et al. 2011) and CXCL12 abundant reticular (CAR) cells (Omatsu et al. 2010) leptin receptor positive (Ding et al. 2012), nestin

positive (Méndez-Ferrer et al. 2010) or platelet-derived growth factor receptor (PDGFR)-α^+ (Morikawa et al. 2009) mesenchymal stromal cells (MSC)) and hematopoietic cells (e.g. macrophages (Chow et al. 2011; Winkler et al. 2010) and megakaryocytes (Shen & Nilsson 2012; Bruns et al. 2014; Zhao et al. 2014; Nakamura-Ishizu et al. 2015; Nakamura-Ishizu, Takubo, et al. 2014)).

These different type of BM niche cells in different BM locations produce a variety of molecular niche factors to control steady state HSC homeostasis, i.e., retention/migration, quiescence/cycling through ligand to cell surface receptor interactions. Examples are Ang-1/Tie2 (Arai et al. 2004), CXCL12/CXCR4, SCF /c-Kit (Sugiyama et al. 2006; Ding & Morrison 2013; Ding et al. 2012), TPO/cMPL (Yoshihara et al. 2007), tumor growth factor (TGF)-β/Endoglin (Yamazaki et al. 2011), Wnt/Frizzle (B. Li et al. 2010; Sugimura et al. 2012), N-cadherin (Sugimura et al. 2012; Hosokawa et al. 2010; Zhang et al. 2003), and E-selectin (Winkler et al. 2012).

Hematopoietic Stem and Progenitor Cells heterogeneity

Recent studies with single cell transplantation and lineage tracing have revealed cellular heterogeneity within functionally-defined HSC populations (Dykstra et al. 2007; Morita et al. 2010; Yamamoto et al. 2013; D. J. Rossi et al. 2005; Beerman et al. 2010; Sanjuan-Pla et al. 2013; Oguro et al. 2013). These studies identified subtypes of HSCs that have distinct lineage differentiation potentials: myeloid-biased HSCs that give rise to more myeloid lineage than lymphoid lineage; lymphoid-biased HSCs that preferentially produce lymphoid lineage cells; and balanced HSCs that show equal production of myeloid and lymphoid lineage (Dykstra et al. 2011). It was indicated that there is a relationship between myeloid biased and lymphoid biased HSCs (Morita et al. 2010): myeloid-biased HSCs defined as $CD150^{high}CD34^-$ LKS have greater self-renewal potential than other HSCs, and are able to give rise to all types of HSCs in transplantation settings, indicating that they are the most primitive population within the HSC hierarchy. Also, it has been found that a subset of HSCs that express von Willebrand Factor (vWF), efficiently gives rise to platelets and erythroid lineage earlier than to other lineages, and therefore are named as platelet-biased HSCs (Sanjuan-Pla et al. 2013).

A recent study by Nina Cabezas-Wallscheid et al. and Pietras et al. (Cabezas-Wallscheid et al. 2014; Pietras et al. 2015) described that there is a certain heterogeneity in multipotent progenitors (MPPs) in terms of their lineage preferential output. Similar studies conducted by Paul et al. (Paul et al. 2015) indicated a more complex relationship between myeloid progenitors CMP/GMP/MEP. Authors identified, based on lineage specific transcription factor expression, at least 5 independent progenitor types that can be found in CMPs.

However it remains to be determined whether HSC subsets that are biased for particular lineage output have any relation to MPP subsets, in particular if lineage biased HSCs are responsible for giving rise preferentially to specific MPP subsets biased for respective lineage output, and whether this can be influenced by stress conditions like inflammation or ageing.

Overall these studies indicate that the lineage choice is predetermined at earlier stages of the hematopoietic tree than it was previously thought.

Proliferative rate of hematopoietic stem and progenitor cells

It has been estimated that a healthy human individual (70kg) produces $490x10^9$ mature blood cells per day (Ogawa 1993; Fliedner et al. 2002). Thus the hematopoietic system is highly proliferative. Neonatal and early post-natal HSCs are actively cycling, with almost 95% HSCs being in cell cycle (Bowie et al. 2006; Nygren et al. 2006). In contrast, most of adult HSCs are, quiescent in steady state and the HSC compartment has a very low turnover rate (about once in a months in mice (Wilson et al. 2008; Nakamura-Ishizu, Takizawa, et al. 2014; Takizawa & Manz 2011)). It has been demonstrated that 80-95% of HSCs are in G0/G1 of cell cycle in an adult mice (Kiel et al. 2007; Passegué et al. 2005; Laurenti et al. 2015). Daily hematopoietic cell production is sustained by lineage committed hematopoietic progenitor cells (HPC) that have a high proliferative rate, with 58-80% being in G0 phase and 20-38% being in S/G2/M phase (Sanjuan-Pla et al. 2013; Busch et al. 2015; Passegué et al. 2005).

The fact that HSCs are found in quiescent and cycling cell phases indicates the possibility that 2 subpopulations co-exist: quiescent HSCs and active cycling HSCs. When label retaining methods were employed to study the proliferative history and HSC cell

turnover, several models of HSC cycling were proposed (Wilson et al. 2008; Takizawa et al. 2011):

a) a clonal succession model, where active cycling HSCs contribute to hematopoiesis until they fully differentiate and loose self-renewal (Foudi et al. 2009; Wilson et al. 2008; Qiu et al. 2014); this model might indicate the presence of HSCs that never contributed to hematopoiesis throughout life;

b) a dynamic variation model (Figure 3) in which HSCs reversibly switch their cycling status over time, ensuring that all HSCs equally contributed to blood production over the lifetime of an individual.

The clonal succession model might be partially criticized by the fact that telomere shortening (as relative measurement of HSC cellular division) happens with ageing and is in addition relatively homogenously distributed in the peripheral blood granulocytes (Vaziri et al. 1994). The clonal succession model also implies that with age the number of HSC will be decreased, although experimental phenotypic and functional data indicate the opposite (discussed in details below).

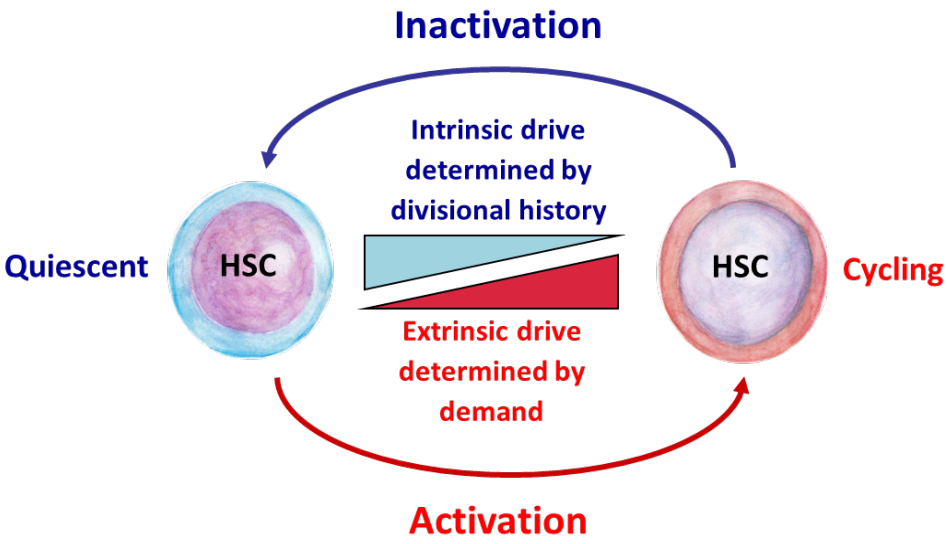
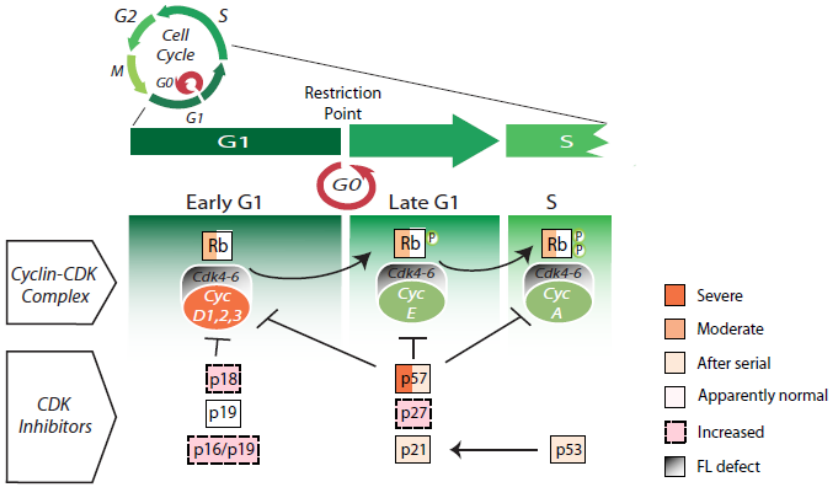


Figure 3. Dynamic cycling activity of HSCs. Figure adapted from (Takizawa and Manz, Cell Cycle 2011)

The HSC cell cycle is regulated by a family of cell cycle regulators: Cdks kinases that drive cell cycle progression and Cdk inhibitors CKIs, which blunt the cell cycle progression (Pietras et al. 2011). Loss of function studies in particular cell cycle regulators unveiled the complex network of interaction of Cdk and CKI in HSCs. Remarkably, when single KO for Cdk or CKI were analyzed, no or little phenotype in the hematopoietic system was observed,

most likely due to existing compensatory mechanisms (Zou et al. 2011), whereas double or triple KOs demonstrated significant dysfunction in HSC phenotype and function.



Gene	Type of Mouse	Primary Hematopoietic Defect	Engraftment Ability in Transplantation Analysis: Severe Defect	Moderate Defect	Mild Defect	Defect after serial transplant	Apparantly Normal	Increased	Cells Tested	Altered Proliferation§ (+: increased)	PMID
Cdc42	cKO	+	+						BM	NA	17702896
Cdkn1c (p57)	cKO	+		+					BM	+	21885021
Cdc42Gap	KO	+			+				FL	NA	16174757
Cdkn1c (p57)	KO	PD				+			FL	NC	21885020
Cdkn1a (p21)	KO	-				+			BM	+	10710306
Cdkn2d (p19Arf)	KO	-					+		BM	NA	15692066
Cdkn1b (p27)	KO	-						+	BM	+	11062534
Cdkn2c (p18)	KO	-						+	BM	+	15122268
Cdkn2a/Cdkn2d (p16Ink4a/p19Arf)	KO	-						+	BM	NA	15692066

Figure 4. Cell cycle regulators in HSCs. Figure and following figure legend taken from (L.Rossi Cell Stem Cell Review 2012.) Cell cycle regulators are crucial for HSC maintenance and regulate quiescence and self-renewal. Box color indicates the degree of hematopoietic defect evident from KO studies. All indicated phenotypes are derived from in vivo transplantation experiments. KO: knockout; cKO: conditional KO; Defect after serial transplant: normal hematopoiesis after primary transplant, but defects emerge after secondary or tertiary transplant; Increased function: KO mice that showed enhanced PB production after HSC or BM transplantation; PMID: PubMed ID number; PD: Perinatal Death, NA: Not Analyzed, NC: No Change, BM: Bone Marrow, FL: Fetal Liver; § proliferation phenotypes in hematopoietic progenitors (not always homogeneous HSCs).

When depleting cell cycle inhibitors, a consistent increased cell cycle activity was often observed, which progressively lead to loss of self-renewal capacity of the HSCs: p57 and p27 double KO (Zou et al. 2011), or p57 alone in another study demonstrated decreased functionality in lethal irradiation and subsequent transplantation settings (Matsumoto et al. 2011). On the contrary, p18 deletion alone lead to increased cell cycle entry, without loss of

self-renewal capacity of HSCs, and with even increasing competitive advantage in irradiation transplantation settings (Yuan et al. 2004). Multiple knock outs of cyclins D4/6 -/- and cyclins D1/2/3 -/- resulted in impaired cell cycle progression (cell cycle blockade) and demonstrated embryonic lethality (Kozar et al. 2004; Malumbres et al. 2004), emphasizing the role of cell cycle turnover during the HSC expansion phase in embryogenesis. These studies were recently summarized and reviewed (Pietras et al. 2011; L. Rossi et al. 2012). A summary is demonstrated in the Figure 4 (L. Rossi et al. 2012).

Importantly, the cell cycle regulators in HSCs do not function completely independent, i.e., they can be stimulated or inhibited extrinsically through growth factors, cytokines, or respective pathogen recognition receptors, that subsequently activate master regulators like STAT5a/b (Wang & Bunting 2013; Hennighausen & Robinson 2008), Phosphatidylinositol-3 kinase (PI3K), or serine/threonine protein kinase B (also known as Akt) and mammalian target of rapamycin (mTOR). These master regulators subsequently can activate/inhibit cell cycle regulators.

Hematopoietic Stem Cell response to stress

Hematopoietic stem and progenitor cells (HSCs/HPCs) are equipped with cell surface receptors for different cytokines and pathogens (pathogen associated molecular pattern recognition receptors), and thus can integrate external signals, that are produced or released locally or systematically (Manz & Boettcher 2014), and convert them into HSC signaling (Zhao et al. 2014). This signaling can lead to demand-adapted hematopoiesis (Takizawa et al. 2012) by enhancing proliferation and differentiation.

For example, hematopoietic stress such as bleeding and infection can activate the hematopoietic system to increase proliferation rates massively to maintain hematopoietic homeostasis as demonstrated by Cheshier et al (Cheshier et al. 2007). Inflammation is defined as an adaptive response, underlined by a variety of physiological and pathological processes that are in part caused by infection and tissue injury/damage and bleeding (G. Y. Chen & Núñez 2010). Professional immune effector cells in both innate and adaptive immunity are short-lived (except lifelong-maintained naïve and memory B and T cells). Upon their consumption during inflammation, these need to be regenerated by HSC and HPCs from BM (Takizawa et al. 2012). Studies by Zhao et al. (Zhao et al. 2014) demonstrate that

upon ligation of TLR-2 and -4, HSC/HPCs are able to secrete pro-inflammatory cytokines, e.g., IL-6, TGF-β, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF), that promote myelopoiesis in a para- or auto-crine fashion (Figure 5). Interestingly, the production of cytokines and growth factors at the single cell level was higher by breadth and magnitude in HSC/HPCs compared to mature hematopoietic cells. However, taking into account that mature cells numerically vastly dominate over HSC/HPCs, it is likely that mature cells in sum are the main producers of inflammatory cytokines upon infection. Since HSC/HPCs also express a broad spectrum of inflammatory cytokine and chemokine receptors (Takizawa et al. 2012), they can detect the milieu of pro-inflammatory signals via, which are systemically or locally released by activated immune cells (Essers et al. 2009; Pietras et al. 2014; Sato et al. 2009; Baldridge et al. 2010).

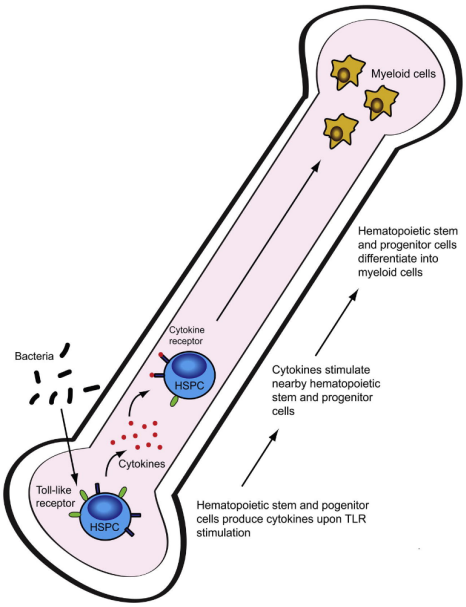


Figure 5. Hematopoietic stem and progenitor cells directly respond to TLR ligand stimulation and convert this signal to subsequent cytokine signaling. Not only mature immune cells but HSC/HPCs as well can respond to bacterial infection by secreting inflammatory cytokines, which in turn can stimulate other cells in autocrine and paracrine fashion ultimately resulting in increased myelopoiesis. (Figure taken from J. L. Zhao et al. 2014)

Persistent inflammatory response of HSCs/HPCs leads to HSC dysfunction and exhaustion, resembling a state that is similarly detected in HSCs with increased turnover rates. Recent studies also have demonstrated that sustained IFN-α/γ activation during chronic infection impairs HSC function, and causes BM failure (Baldridge et al. 2010; Walter et al. 2015). Interestingly, chronic TNF-α signaling is associated with myelodysplastic syndrome (MDS) and bone marrow failure (Kitagawa et al. 1997). Therefore, it seems mandatory that inflammatory responses are terminated when no longer needed, as continuous signaling might lead or at least support hematopoietic diseases (Kristinsson et al. 2010).

Ageing of the hematopoietic system

Ageing of hematopoietic system is accompanied by a functional decline in various hematopoietic lineages – from erythroid lineage to both the adaptive and innate immune system. It can be illustrated by several observations:

a) A decreased diversity of the B cell repertoire is accompanied during ageing by decreased antibody affinity and impaired class switch recombination. In addition, B cells are prone to produce auto-antibodies, increasing the incidence of spontaneous autoimmunity (Frasca et al. 2008; Dorshkind et al. 2009);

b) De novo T cell production also declines with ageing partially due to thymic involution. CD8+ T cells undergo oligoclonal expansion and their repertoire is skewed to previously encountered antigens (Akbar & Fletcher 2005) (Figure 6);

c) Natural killer (NK) cells have diminished cytotoxicity and cytokine secretion;

d) Despite increase in number of myeloid cells (Figure 6), their functionality is decreased, and e.g. neutrophils show reduced migration in response to stimuli, and macrophages display reduced phagocytic activity and decreased oxidative burst (Ogata et al. 1997; Kuranda et al. 2011; Plowden et al. 2004);

e) Erythropoiesis also declines in elderly people, leading frequently to anemia (Berliner 2013), while the thrombocyte lineage has not been reported so far to be significantly affected during ageing.

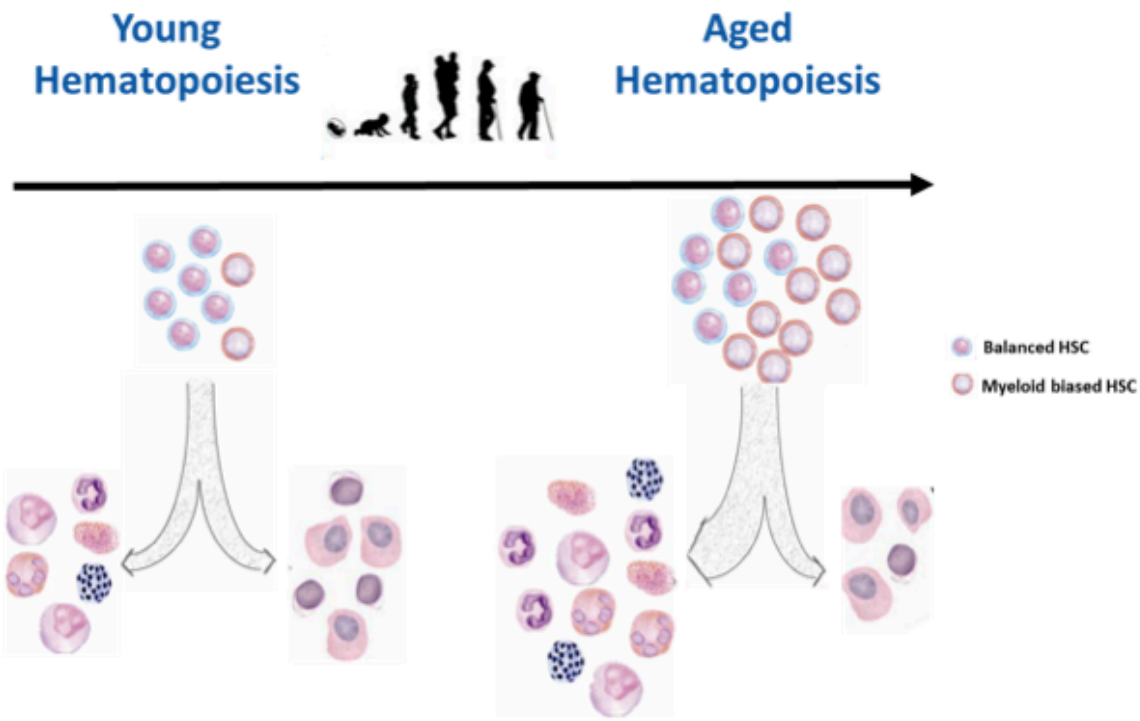


Figure 6. Ageing of Hematopoietic system and HSCs

Indeed, ageing associated immunosenescence leads to increased susceptibility to infections, low efficacy of vaccination, and increased vulnerability to development of autoimmunity and hematopoietic malignancies (Haq & McElhaney 2014).

Hematopoietic stem cell ageing

Since multiple blood lineages are affected during ageing, it is likely that ageing of the hematopoietic system initiates at the HSC level. In fact there is a specific ageing phenotype of HSCs that is defined by the following alterations:

a) An increased number of phenotypically and functionally defined HSC (Figure 6) (Sudo et al. 2000);

b) An increased number of the subpopulation of myeloid-biased HSCs (Figure 6) (Morita et al. 2010; Beerman et al. 2010);

c) A lineage-biased repopulation of the myeloid lineage upon transplantation, which persists in serial transplantation settings (Beerman et al. 2010; Dykstra et al. 2011; Harrison & Astle 1982) (indicative of cell autonomous changes upon ageing) and also involves

disturbance in the committed progenitors – aged mice have a decreased frequency of CLPs and increased frequencies of GMPs, whereas CMPs remain unaffected;

d) A reduced self-renewal capacity of HSCs on per cell basis (Sudo et al. 2000; Dykstra et al. 2011; Morrison et al. 1996; Chambers et al. 2007);

e) A reduced homing efficiency to the bone marrow when transplanted intravenously into lethally irradiated mice (Dykstra et al. 2011), although the mobilization by G-CSF is similar to young HSCs.

These characteristics of HSC ageing can be in part experimentally recapitulated by increasing the proliferative history by stress of HSCs with multiple injections of myeloablative chemotherapeutic regimens like 5FU (Beerman et al. 2013) or with serial rounds of transplantation (Harrison & Astle 1982; Dykstra et al. 2011) ('experimental ageing'). While it is clear that both experimental ageing settings push HSCs towards proliferation, it is not clear how much inflammation induced by both settings (5FU administration and irradiation induced inflammation) contributes to the acquired ageing phenotype.

Several groups had compared cycling activity of young versus aged HSCs. The results are, however, rather controversial: some indicate that aged HSCs have increased cycling activity (Morrison et al. 1996), whereas some suggest no difference in cell cycling activity (Sudo et al. 2000; Chambers et al. 2007). Moreover, others reported a more quiescent status of aged HSCs compared to young HSC (C. Chen et al. 2009; Takizawa & Manz 2011). These contradictions might be partially explained by different definition of HSCs and different experimental settings to measure the cell cycle status (reviewed in (Nakamura-Ishizu, Takizawa, et al. 2014)).

However it remains unclear whether the age-associated myeloid bias that occurs in aged HSC population is the result of clonal evolution or a population shift, i.e. an age-dependent decline of lymphoid biased HSCs with less self-renewal potential (Morita et al. 2010) and an expansion of myeloid biased (Beerman et al. 2010) or platelet biased HSCs (Sanjuan-Pla et al. 2013), or if it is a result of functional changes occurring in all individual HSC clones with different lineage output (Dykstra et al. 2007; Dykstra et al. 2011). Moreover, the recent identification of myeloid restricted progenitors with long term repopulating capacity/self-renewal questions the definition of HSCs (Yamamoto et al. 2013; Haas et al. 2015)

The shift from lymphopoiesis to myelopoiesis might also be a result of increased levels of pro-inflammatory cytokines, such as IL-6, TNFa, IL-1 and IL-1ra (Haq & McElhaney 2014; Baylis et al. 2013; Ferrucci et al. 2010) that are observed in the elderly population, a situation also called “inflammageing”. The consequence of this inflammageing is production of reactive oxygen species, and release of more inflammatory cytokines, creating thereby a feedback loop to support a state of low-grade chronic inflammation.

Potential mechanisms of Hematopoietic Stem Cell ageing

Previous studies demonstrated that ageing of HSCs is influenced by both cell-intrinsic and cell-extrinsic effects. Transcriptome profiles from aged and young HSCs have provided some insights on what potential molecular mechanisms could be involved in HSC ageing. Overall, aged HSC are reported to have up-regulated stress response, up-regulated inflammatory signaling and protein misfolding, and down-regulated DNA repair machinery and aberrant chromatin modification (Chambers et al. 2007). And indeed, further investigation into these processes demonstrated that aged HSCs accumulate DNA damage (D. J. Rossi et al. 2007; Rübe et al. 2011), possibly due to a higher level of intracellular ROS (Ito et al. 2006). Other studies indicated accumulation of proliferative stress markers in aged HSCs (as measured by increased numbers of γH2AX foci) that cause inefficient DNA replication and transcriptional repression (Flach et al. 2014). Aged HSCs also exhibit activated mammalian target of rapamycin (mTOR) (C. Chen et al. 2009), have activated an autophagy-dependent survival program (Warr et al. 2013), dysregulated DNA methylation (specifically at the site of genes controlling myeloid and lymphoid balancing (Beerman et al. 2014)), and impaired histone modification (Sun et al. 2014). HSCs also exhibit disturbed cell polarity and distribution of Cdc42 in the cytoplasm (Florian et al. 2012).

HSCs are preserved in the bone marrow niche, which physically likely holds a protective role, but also supplies HSCs with soluble and membrane-bound factors that maintain their function. The HSC niche is also affected by ageing. It has been shown that niche composition is influenced by enhanced adipogenesis (Justesen et al. 2001), possibly linked to dysregulation in insulin growth factor 1 (IGF-1) signaling (Linton & Dorshkind 2004), changes in extracellular matrix (ECM) components and decreased bone formation (Wagner et al. 2008; Bellantuono et al. 2009). The aged environment is also thought to be more

supportive for myelopoiesis, and the young environment more supportive for lymphopoiesis. In settings when HSCs were transplanted into lethally irradiated young or aged environments, HSCs in the aged environment tend to produce slightly more myeloid cells then in a young environment (D. J. Rossi et al. 2005; Ergen et al. 2012).

Rejuvenation of aged Hematopoietic Stem Cells

Several groups addressed the question whether an aged phenotype can be pharmacologically reversed by inhibition of specific intrinsic and extrinsic mechanisms:

a) Restoring the cell polarity of aged HSCs: Cdc42 is distributed in a polarized manner in young HSCs, whereas in aged HSCs this polarity is disoriented. Pharmacological inhibition of Cdc42 activity functionally rejuvenates aged HSCs, and increases the percentage of polarized cells in an aged HSC population (Florian et al. 2012).

b) In aged HSCs mTOR activity is increased, and pharmacological inhibition of mTOR by rapamycin restored the functionality of aged HSCs and increased lifespan of mice, as demonstrated by two independent studies (C. Chen et al. 2009; Harrison et al. 2009).

c) IGF-1 inhibition: prolonged fasting reduces systemic age-associated increase in IGF-1, prevents formation of an experimentally aged phenotype of HSCs (in combination with chemotherapy), and reverses the age-associated myeloid bias in 18 month old mice (Cheng et al. 2014); specific local inhibition of IGF-1 in the osteoblastic niche also lead to rejuvenation of HSCs (Mayack et al. 2010).

The fact that the HSC phenotype can be reversed indicates that it is more likely epigenetic mechanisms and/or protein folding or functional signaling changes that are the driving cause of HSC ageing, and that the ageing phenotype is not primarily driven by accumulating DNA mutations.

Ageing-associated inflammation „inflammageing“

There are some similarities in hematopoietic cell function in inflammation and ageing. Upon both ageing and inflammation induced via bacterial infection, myelopoiesis favors over lymphopoiesis (Sudo et al. 2000; Morrison et al. 1996; Kim et al. 2003; Esplin et al. 2011). Also, B-lymphopoiesis is impaired due to decreased level of E47, a transcription

factor essential for B cell development, in aged (Van der Put et al. 2004) and LPS treated mice (Esplin et al. 2011). The aging associated myeloid dominance might result in increased basal levels of pro-inflammatory cytokines, even in the absence of infection. Indeed, it has been reported that the level of circulating pro-inflammatory cytokines, such as IL-6, TNF-α, IL-1Ra, C reactive protein, are up-regulated in the elderly population (Haq & McElhaney 2014; Baylis et al. 2013; Cohen et al. 1997; Hasegawa et al. 2000; Bruunsgaard et al. 1999; Emerging Risk Factors Collaboration et al. 2010; Ferrucci et al. 2005; Ferrucci et al. 2010). These observations propose a hypothesis of "inflamm-ageing": a chronic sub-clinical inflammatory status might contribute to initiation or acceleration of hematopoietic ageing. Ageing-associated phenotypes of HSCs are similar to the function of HSCs exposed to chronic inflammatory stimuli, including a reduced self-renewal and myelopoiesis-favored differentiation potential. Accordingly, the HSC pool is shifted to increased proportion of $CD150^{high}$ HSC that predominantly produces myeloid lineage cells (Beerman et al. 2010; Challen et al. 2010) when mice were treated daily with a low dose of LPS over a month (Esplin et al. 2011). Recent studies have suggested that accumulation of myeloid-skewed HSCs with ageing is regulated by activated signaling of TLR4 (Esplin et al. 2011), P-selectin (Chambers et al. 2007), nuclear factor kappa beta (NFκB) (Chambers et al. 2007), RANTES-mTOR pathway (C. Chen et al. 2009; Ergen et al. 2012), and TGF-β (Challen et al. 2010). Since all these pro-inflammatory cytokines are produced by myeloid cell lineages, there might be a positive feedback mechanism by which HSCs with myeloid lineage skewing are accumulated upon ageing and give rise to more myeloid cells that produce pro-inflammatory cytokines that further expand themselves and advance myeloid dominancy. Moreover, a pro-inflammatory state promotes production of ROS in hematopoietic cells, i.e. genotoxic reagents that are known to cause DNA damage (Ito et al. 2006; C. Chen et al. 2010; C. Chen et al. 2009). In fact, it has been shown that DNA damage is induced by intracellular ROS production in HSC and accumulated with viral infection and ageing (D. J. Rossi et al. 2007; Walter et al. 2015), although another study did not observe DNA damage in aged HSCs (Flach et al. 2014), but rather correlates of proliferative stress.

STUDY AIMS

There is evidence that hematopoietic stem cells are able to respond to stress and demand by active proliferation, however taking into account that stress induced cytokine and growth factor spectra is quite broad, it is important to understand the role of each cytokine, chemokine, growth factor and hematopoietic factor on HSCs.

Aim 1. To evaluate the impact of enhanced cytokine challenge on HSC division rate and function.

Several scenarios are possible as mechanism of HSCs activation to proliferation by stimulating factors: 1.) a direct receptor/ligand interaction if the respective receptor is expressed on HSCs; 2.) an indirect interaction by stimulating progenitor cells that express the respective receptor and induce HPC proliferation, thereby emptying the HPC pool, leading to a "pull down" of HSCs to replenish HPC pools; 3.) an induction of proliferation via stimulation of third party cells that then produce HSC proliferation factors

A particular interest of this aim was to dissect the impact of clinically applied cytokines and a chemokine receptor antagonist, i.e. those hematopoietic factors that are already exploited in clinical medicine. We thus selected the following factors:

a) Romiplostim, a thrombopoietin mimetic (Nplate), further called cMpl agonist, applied to patients with immune thrombocytopenia or aplastic anemia (Kuter et al. 2010);

b) Recombinant G-CSF (Filgrastim), applied to patients with neutropenia, or as agent for mobilization of HSC/HPCs for autologous or allogeneic hematopoietic stem cell transplantation (HSCT) (Talhi et al. 2013; Hoggatt & Pelus 2014);

c) An antagonist of CXCR4 (Plerixafor), also used to mobilize HSPCs for autologous or allogeneic HSCT (Liles et al. 2003).

d) Flt3L was selected in addition to specifically test the hypothesis of a "pull-down" effect, since the Flt3 receptor is not expressed at the HSC level but it is highly expressed at all early HPCs.

An ageing associated HSC phenotype has been well described in the past decades. Yet the mechanisms inducing an ageing phenotype remain descriptive. Specifically, it remains unclear how intrinsic and extrinsic factors influence self-renewal, differentiation and

turnover of HSCs during ageing, how these factors contribute relatively to HSC cell fate decisions, what the underlying molecular mechanisms and signatures are, and whether ageing can be rejuvenated as a measure for regenerative medicine. These questions were addressed by setting the following specific aims:

Aim 2: To dissect the relative contribution of cell-intrinsic and -extrinsic factors in HSC division and cell fate decision during ageing on a population basis and on the single cell level.

Aim 3: To determine systemic and/or local BM specific ageing-associated cell-extrinsic factors and to dissect their contribution to the ageing phenotype.

RESEARCH ARTICLE 1

(Submitted to Blood as Brief Report, and currently in revision)

ENHANCED THROMBOPOIETIN BUT NOT G-CSF RECEPTOR STIMULATION INDUCES SELF-RENEWING HEMATOPOIETIC STEM CELL DIVISIONS IN VIVO

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*Hitoshi Takizawa performed 1 out of 14 experiments for Figure 1D, rest was performed by Larisa Kovtonyuk.

Key points:

- cMpl agonist but not G-CSF induces self-renewing HSC divisions
- cMpl agonist sensitizes HSPCs and possibly LICs to chemotherapeutic agents

ABSTRACT

In steady-state adult hematopoiesis, most hematopoietic stem cells (HSCs) are in G0 phase of the cell cycle. Upon enhanced hematopoietic demand, HSCs can be induced to divide and self-renew or differentiate. However, which cell-extrinsic signals induce HSC cycling remains to be determined. Using in vivo high-resolution single HSC divisional tracking, we here directly demonstrate that clinically applied thrombopoietin (TPO) receptor but not granulocyte-colony stimulating factor receptor (G-CSFR) agonists drive HSCs into self-renewing divisions and lead to quantitative expansion of functional HSC as defined by their in vivo serial multi-lineage long-term repopulating potential. These results suggest that TPO mimetics might be used to expand HSCs in vivo, and furthermore to sensitize normal and possibly malignant, TPO receptor-expressing HSCs to cell cycle toxic agents.

INTRODUCTION

During steady-state hematopoiesis 80-90% of HSCs are quiescent in G0 of cell cycle (Passegué et al. 2005; Laurenti et al. 2015; Wilson et al. 2008; Takizawa et al. 2011). Thus, at any given time, steady-state hematopoiesis is sustained by a small fraction of HSCs and downstream HPCs(Busch et al. 2015; Sun et al. 2014). However, upon demand, e.g. massive blood loss or severe infection, blood production can be enhanced several-fold (Passegué et al. 2005; Laurenti et al. 2015; Wilson et al. 2008; Takizawa et al. 2011; Takizawa et al. 2012; Manz & Boettcher 2014; King & Goodell 2011; Christensen & Weissman 2001). While some of this is accomplished by HPCs, quiescent HSCs are also temporarily recruited into cell cycle(Wilson et al. 2008; Takizawa et al. 2011; Busch et al. 2015; Sun et al. 2014; Takizawa et al. 2012). Identification of factors that trigger HSC cycling without causing HSC loss might inform on new therapeutic interventions. However, to determine HSC self-renewal factors that are altered upon hematopoietic demand remains technically challenging due to the difficulty to dissect HSC divisional behavior and function in vivo. We recently established an in vivo assay allowing high-resolution single HSC divisional tracking, and subsequent assessment of HSC biology by serial transplantation(Takizawa et al. 2011). We here used this to address the long-standing question of how the clinically-employed agonists to granulocyte colony-stimulating factor receptor (G-CSFR) (Filgrastim), to fetal liver tyrosine kinase 3 (Flt3-ligand) and to thrombopoietin (TPO) receptor (cMpl) (Romiplostim), as well as an antagonist to chemokine receptor CXCR4 (Plerixafor), affect in vivo maintenance and proliferation of bona fide HSCs.

METHODS

Mice

C57BL/6J (CD45.2$^+$) and B6.SJL (CD45.1$^+$) were obtained from Jackson Laboratories, and both were intercrossed to generate CD45.1/2$^+$ mice. All mice were maintained in University Hospital Zurich animal facility according to the guidelines of the Swiss Federal Veterinary Office, and all experiments were approved by the Veterinärmt Kanton Zurich, Zurich, Switzerland.

CFSE chasing, drug challenge, analysis and serial transplantation

Lin-c-Kit+Sca-1+ cells (LKS) were FAC-sorted, labelled with CFSE (5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester) and i.v. transplanted as described previously(Takizawa et al. 2011). One week after transplantation mice were injected daily i.p. with either PBS, G-CSF (7.5 µg/mouse), cMpl-agonist (2.5 µg/mouse), Flt3-ligand (30 µg/mouse), or s.c. with CXCR4-antagonist (125 µg/mouse). Donor cells were sorted based on CFSE-dilution-defined divisional history one week after final injections (i.e. 3 weeks after initial transfer) and transplanted into lethally irradiated secondary recipients. Four month later, whole BM cells were serially transplanted into lethally irradiated tertiary recipient mice.

SUPPLEMENTAL METHODS

Quantitative RT-PCR

HSC and HPC from BM were enriched for Lin- cells with PE-Cy5 conjugated lineage markers as described above. Cells were then stained with the following antibodies: FITC-conjugated FcγR (93), PE-Cy7-conjugated antibody to c-Kit (2B8), APC-Cy7-conjugated antibody to Sca-1 (D7), biotinylated antibody to cMpl (AMM2), Qdot605-conjugated streptavidin, PE-conjugated antibody to Flt3 (A2F10), eFluor450-conjugated antibody to CD48 (HM48-1), Alexa Fluor 660-conjugated antibody to CD34 (RAM34), Brilliant Violet 510 conjugated antibody to CD150 (TC15-12F12.2). For mature cell populations, BM cells were stained with Alexa Fluor 780-conjugated antibody to Mac1 (M1/70) and PE-conjugated antibody to Gr-1. Spleen cells were stained with PE-Cy5-conjugated antibody to CD3ε (145-

2C11), PE-Cy7-conjugated antibody to CD19 (1D3), FITC-conjugated antibody to MHCII (M5/114.15.2), APC-conjugated to CD11c (N418). $1x10^4$ cells were directly sorted into lysis buffer with RNeasy Plus Micro Kit (Qiagen) containing β-mercaptoethanol. Cells were subjected to RNA isolation (Qiagen RNeasy Plus Micro Kit), cDNA synthesis (Applied Biosystems) and qPCR with TaqMan Assays-on-Demand probes on 7500 Fast Real Time PCR System (Applied Biosystems). qPCR for *Csf3r, Cxcr4, Mpl* and *Flt3* were performed using TaqMan Assays-on-Demand probes (Csf3r: Mm00432735_m1, Cxcr4: Mm01996749_s1, Mpl: Mm00440310_m1, Flt3: Mm00438996_m1 (all from Applied Biosystems)) on 7500 Fast Real Time PCR System (Applied Biosystems). Relative mRNA expression of each gene was calculated against expression of beta-actin (TaqMan probe: Mm00607939_s1). All antibodies were obtained from BioLegend, eBioscience or Life technologies., except anti-cMpl antibody (AMM2) that was kindly donated by Kyowa Hakko Kirin Co., Ltd. (Japan)

CFSE labeling, mouse treatment and CFSE chasing

After whole BM cells were harvested from hip bones, femurs, tibias and spine of $CD45.2^+$ mice, red blood cells were lysed with lysis buffer (150 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA). For LKS sorting, cells were stained with biotinylated antibodies against the lineage antigens B220 (RA3-6B2), CD3ε (145-2C11), Ter119 (Ter119), NK1.1 (PK136), CD11b (M1/70), CD4 (RM4-5), CD8α (53-6.7), IL-7Rα (A7R34) and Gr-1 (RB6-8C5), and immune-magnetically enriched for lineage negative cells using streptavidin microbeads (Miltenyi Biotec). The lineage-depleted (Lin^-) cells were subsequently labeled with pacific blue-conjugated streptavidin, PE-Cy5-conjugated anti-c-Kit (2B8) and APC-Cy7-conjugated anti-Sca-1 (D7). $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ (LKS) were sorted using a FACSAria III (BD Biosciences). $CD4^+CD62L^+$ (naïve T) cells were enriched from spleen cells with biotin-conjugated anti-CD4 antibody (GK1.5) and streptavidin microbeads and stained with PE-Cy5-conjugated streptavidin and PE-conjugated anti-CD62L antibody (MEL-14) and sorted on a FACSAria III. LKS and naïve T cells were labelled with 2 µM CFSE (Invitrogen), and $1.0\text{-}1.1x10^5$ LKS or $1.5x10^6$ T cells ($CD45.2^+$) were i.v. transplanted into non-irradiated $CD45.1/2^+$ mice. One week after transplantation mice were injected daily i.p. with PBS (x7 injections), 7.5 µg/mouse G-CSF (x5 injections, Filgrastim, Neupogen), 2.5 µg/mouse cMpl agonist (x7 injections, Romiplostim, Nplate, Amgen), 30 µg/mouse Flt3L, and s.c. with 125 µg/mouse CXCR4 antagonist (x3 injections, Plerixafor, Genzyme Europe).

FACS analysis/sorting and serial transplantation

For analysis and sorting, 1 week after the final injection and 3 weeks after primary transfer and CFSE chasing, Lin- cells were enriched from whole BM cells with PE-Cy5-conjugated antibodies against lineage antigens listed above, and anti-Cy5/anti–Alexa Fluor 647 microbeads (Miltenyi Biotec) on MACS columns or Easy Sep pour off columns (Stem Cell Technologies). The resultant cells were stained with the following antibodies: PE-Cy7-conjugated antibody to c-Kit (2B8), APC-Cy7-conjugated antibody to Sca-1 (D7), biotinylated antibody to cMpl (AMM2), Qdot605-conjugated streptavidin, PE-conjugated antibody to Flt3 (A2F10), eFluor450-conjugated antibody to CD45.1 (A20), APC-conjugated antibody to CD45.2 (104). The zero-divisional cell fraction was determined according to the CFSE intensity of donor $CD4^+CD62L^+$ as previously described (Takizawa et al. 2011). For transplantation after in vivo chasing, 7-22 donor $CD45.2^+$ LKS $Flt3^-cMpl^+$ were FAC-sorted based on the CFSE-dilution divisional history (0-1x divided, 2-4x divided and >5x divided) and transplanted into lethally irradiated (9.5 Gy) $CD45.1^+$ or $CD45.1/2^+$ mice together with 2.5-$3x10^5$ $CD45.1^+$ whole BM competitor cells. Donor chimerism in PB was followed monthly as previously described (Takizawa et al. 2011): briefly, PB was depleted for erythrocytes with lysis buffer, and stained with the following fluorochrome conjugated antibodies: FITC anti-CD45.1 (A20), PE anti-CD45.2 (104), PE-Cy5 anti-CD3ε (2C11), PE-Cy7 anti-CD19 (1D3), APC anti-Gr-1 (RB6-8C5) and eFluor780 anti-CD11b. For secondary transplantation, $5x10^6$ WBM cells from mice that received sorted cells 4 months before were transplanted into lethally irradiated $CD45.1^+$ or $CD45.1/2^+$ mice. Donor engraftment was scored positive at a threshold of >0.4% donor cell engraftment. HSC frequency was estimated by Poisson statistics.

Absolute counts of donor LKS with different divisional history were calculated by multiplying absolute number of non-enriched total BM cells with the respective percentages of population to divisional LKS groups (0-1x divided, 2-4x divided, 5x and >5x divided cells) within enriched lineage depleted BM.

Survival following 5-FU administration

C57BL/6 mice were i.p. pretreated with PBS, G-CSF or cMpl agonist, according to same injection scheme as for CFSE chasing experiments, followed one day and one week later by each one i.p. injection of 5-FU (Fluorouracil-Teva, Teva) (150 mg/kg). Survival of animals was followed up over one month.

RESULTS AND DISCUSSION

Direct response to a given agonist requires respective receptor expression. We compared the relative mRNA expression of *Csf3r (Gcsfr), Cxcr4, Mpl* and *Flt3* on HSCs, multipotent progenitors (MPPs), and granulocyte-macrophage progenitors (GMP), to mature BM and spleen cells (Figure 7A). The findings extend and are in line with prior reports (Christensen & Weissman 2001; Sugiyama et al. 2006; Yoshihara et al. 2007) and suggest, although not definitively proof, that HSCs can respond directly to cMpl- and to some extend to G-CSFR-agonists and CXCR4-antagonist, but not to Flt3-ligand.

To determine the effects of these receptor agonists/antagonist on HSC proliferation and function, we utilized an in vivo assay, proven to faithfully evaluate HSC divisional behavior (Takizawa et al. 2011). One week after transplantation of CFSE-labeled HSC-containing LKS, recipient mice were injected repetitively with either PBS, or G–CSF, or a CXCR4-antagonist, or a cMpl-agonist, or Flt3-ligand, followed by BM analysis after one week rest, i.e. three weeks after initial LKS cell transfer (Figure 7B). In PBS-injected mice few LKS cells remained in the $CFSE^{hi}$ 0-1x-divided fraction, while increasingly more appeared in the subsequent $CFSE^{low/-}$ 2-4x-, 5x-, and >5x-divided fractions, accompanied by a gradual loss of Sca-1 and c-Kit expression (Takizawa et al. 2011) (Figures 7C-D and supplemental Figure 1). A similar pattern as in PBS injected mice was observed in CXCR4-antagonist receiving mice, while G-CSF injections resulted in relatively less 0-1x-, 2-4x-, and equal or increased 5x- or >5x-divided fractions. In strong contrast, cMpl-agonist injected mice showed very few, if any, 0-1x-divided cells, significantly less or unchanged relative numbers in the 2-4x- and 5x-, and increased numbers in the >5x-divided cellular fraction with compared to other agonist/antagonist injection higher maintenance of Sca-1 and CD150 expression (supplemental Figure 2). Flt3-ligand-injected mice showed unchanged 0-1x-, decreased 2-5x-, and increased >5x-divided cells.

As cMpl-agonist-induced in vivo divisional changes of LKS cells were most striking, we evaluated this in more detail. cMpl-mediated LKS cell proliferation was dose-dependent and led to an increase of divided cells with HSC and MPP immuno-phenotype (supplemental Figure 3). Similarly, cMpl-stimulation expanded phenotypic HSC in mice without prior transfer of LKS cells (supplemental Figure 4). To test if cMpl-agonist injections induce HSPC proliferation, thereby increasing susceptibility to DNA-synthesis targeting drugs (Essers et al.

2009), we co-treated mice with a sub-lethal dose of 5-fluorouracil (5-FU). Indeed, a significant fraction of cMpl-agonist- but not PBS- or G-CSF-treated mice died of hematopoietic failure (supplemental Figure 5).

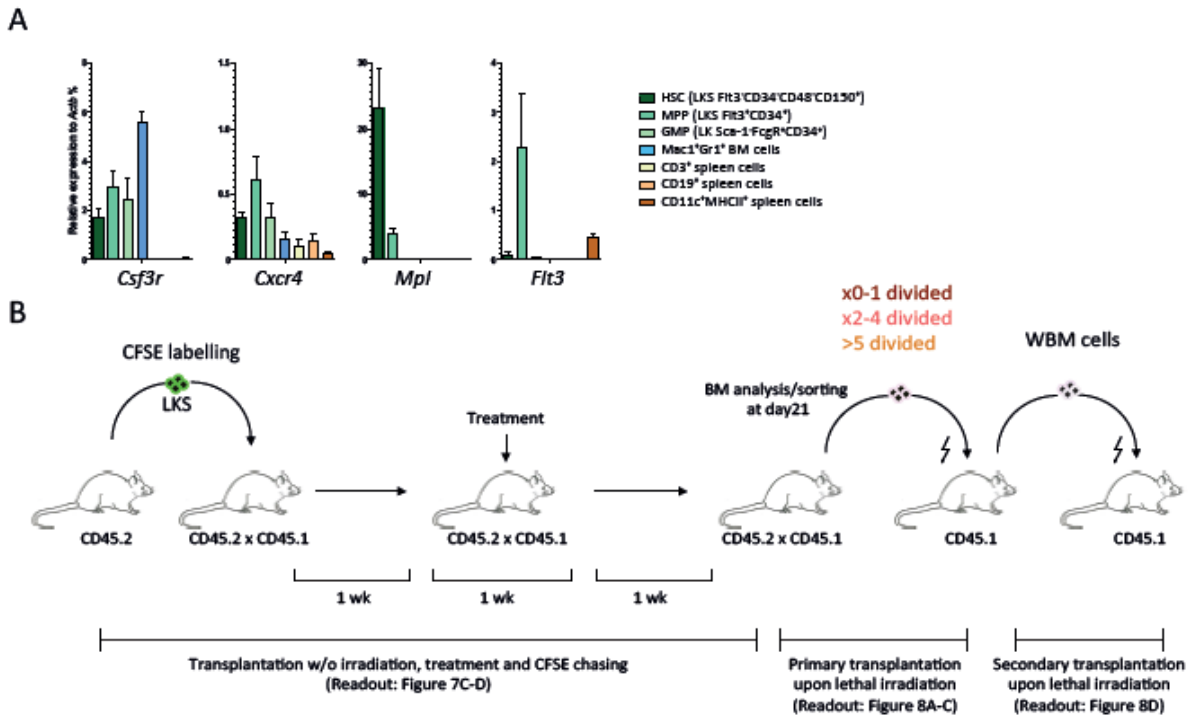
To definitively test whether cMpl stimulation leads to division and expansion of bona-fide, functionally defined HSCs, we sorted 7-22 LKS $Flt3^{-}cMpl^{+}$ cells from each of the 0-1x-, 2-4x- and >5x-divided fractions from PBS-, G-CSF- or cMpl-agonist-treated animals, and transplanted these into lethally irradiated recipient mice (primary transplantation upon lethal irradiation, Figure 7B and 8A). Subsequent monthly donor chimerism analysis revealed long-term multi-lineage repopulating HSCs in 0-1x- (1 in 11.5 cells), much less in 2-4x- (1 in 53.3 cells), and no in >5x-divided cells in PBS-treated mice, confirming prior data on steady-state HSC dormancy (Figure 8B-C, supplemental Figure 6 and Table 1) (Takizawa et al. 2011). Similar results were found in G-CSF-treated animals. Again in strong contrast, cMpl-agonist-treated animals showed high repopulating activity in 2-4x- (1 in 9.4 cells) and still low activity in >5x-divided cells (1 in 178.8 cells). While HSC-defining serial transplantation activity (i.e. transplantation activity in secondary recipients upon lethal irradiation as depicted in scheme Figure 7B) was maintained only in the 0-1x- but not 2-4x-divided fractions from PBS- and G-CSF-treated donors, cMpl-agonist treatment led to transition of serially transplantable HSCs to the 2-4x-divided fraction (Figure 8D). Although the estimated HSC frequency in the 0-1x- and 2-4x-divided populations of PBS- and cMpl-agonist-treated animals, respectively, was almost the same, the total number of functional HSCs was estimated to be 3.8 fold higher in cMpl-agonist-treated animals (Figure 8C and supplemental Figure 1 and Table 1). Thus, cMpl-agonist treatment induced numeric expansion of serially transplantable HSCs.

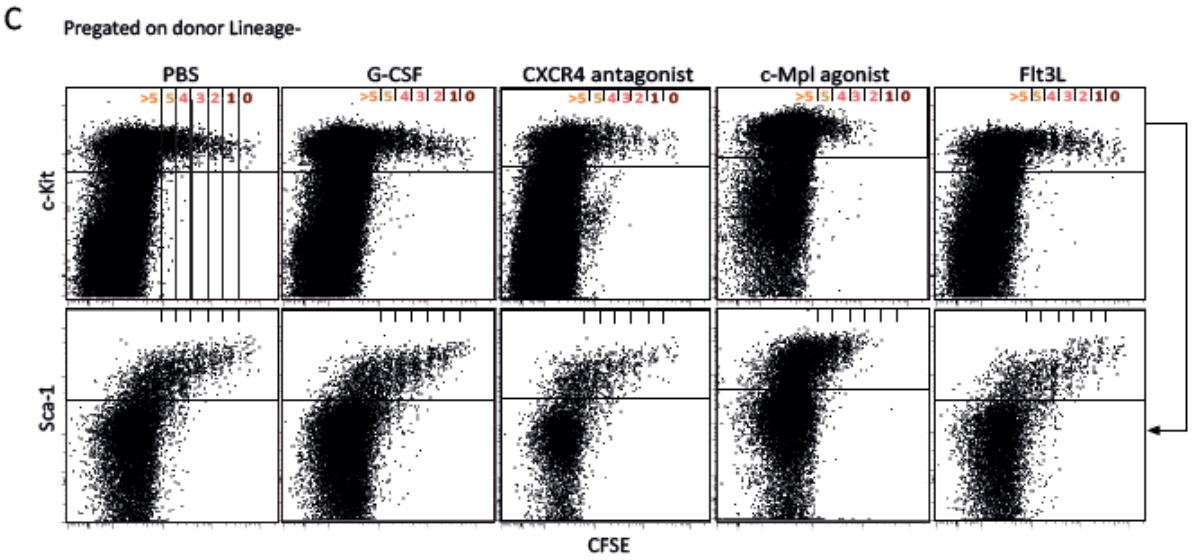
Our and data from others demonstrate that HSCs do not express Flt3 (Christensen & Weissman 2001), explaining lack of Flt3-ligand action on HSCs (Figure 7A). It has been suggested that about 80% of $Rho123^{low}$LKS (containing HSCs with 25% purity) (C. L. Li & Johnson 1995) express G-CSFRα (McKinstry et al. 1997), and G-CSF induces HSC divisions phenotypically (Wilson et al. 2008; Morrison et al. 1997). While confirming low G-CSFR mRNA expression in immune-phenotypic defined HSCs, we did not observe G-CSF-driven division of functional HSC (Figure 7A, C and 8B). This is not a result of insufficient G-CSF dosing (Wilson et al. 2008; Morrison et al. 1997), but rather might be explained by limitations of previous assays to test HSC function. The data presented here together with data on no/low G-CSFR expression on human HSCs (Gibbs et al. 2011) might now explain

why clinical application of G-CSF in overlapping combination with chemotherapy in order to "prime" acute myeloid leukemia cells did not result in HSC loss and permitted normal hematopoietic recovery (Löwenberg et al. 2003).

In contrast to Flt3 and G-CSFR, it has been clearly demonstrated that CXCR4 and cMpl are expressed on HSCs (Sugiyama et al. 2006; Nie et al. 2008; Yoshihara et al. 2007) (Figure 7A and 8A), and that CXCR4 signaling is required to maintain HSC quiescence in adult mice (Sugiyama et al. 2006; Nie et al. 2008). Our data, however, directly demonstrates that HSCs are not activated from dormancy by CXCR4-antagonist application, even at doses that exceed doses used to mobilize HSCs in mice and in humans(Broxmeyer et al. 2005). In strong contrast, we directly demonstrate that cMpl stimulation drives dormant HSCs into division and expansion, a situation that might occur e.g. in sepsis-mediated inflammation (Kaser et al. 2001; Segre et al. 2014). Indeed, prior studies demonstrated that TPO signaling is essential for HSC homeostasis (Qian et al. 2007) and their expansion after transplantation (Fox et al. 2002), whereas, seemingly contradictory, other studies demonstrated that TPO is critical for keeping HSC in a quiescent state (Yoshihara et al. 2007). We suggest that this discrepancy might result from cMpl signaling strength: while data on HSC quiescence stem from loss-of-function studies (Yoshihara et al. 2007), we here examined the effect of supra-steady-state cMpl-signaling, more comparable to studies on higher doses of TPO administration (Yoshihara et al. 2007) or on genetic deficiency of Lnk, a negative regulator of TPO signal transduction (Seita et al. 2007). In addition, as the applied CFSE-dilution assay informs on divisional history of cells, but not on cell cycle status at the moment of analysis, our data does not exclude the possibility that after several divisions, induced upon cMpl activation, HSCs return to G0 and become more quiescent than before.

Given the enhancement of multi-lineage regeneration possibly via re-expansion of human HSCs in aplastic anemia (Olnes et al. 2012) and our data on cMpl-stimulation-mediated HSC sensitization to chemotherapy, we envision that cMpl-agonist treatment might be tested in clinical settings as add-on to conditioning therapy prior to allogeneic HSC transplantation in order to reduce endogenous HSCs, or to sensitize cMpl-expressing myeloid leukemia initiating cells to chemotherapy.


A
Csf3r
Cxcr4
Mpl
Flt3
HSC (LKS Flt3⁻CD34⁻CD48⁻CD150⁺)
MPP (LKS Flt3⁺CD34⁺)
GMP (LK Sca-1⁻FcgR⁺CD34⁺)
Mac1⁺Gr1⁺ BM cells
CD3⁺ spleen cells
CD19⁺ spleen cells
CD11c⁺MHCII⁺ spleen cells
B
CFSE labelling
LKS
Treatment
BM analysis/sorting at day21
x0-1 divided
x2-4 divided
>5 divided
WBM cells
CD45.2
CD45.2 x CD45.1
CD45.2 x CD45.1
CD45.2 x CD45.1
CD45.1
CD45.1
1 wk
1 wk
1 wk
Transplantation w/o irradiation, treatment and CFSE chasing
(Readout: Figure 7C-D)
Primary transplantation
upon lethal irradiation
(Readout: Figure 8A-C)
Secondary transplantation
upon lethal irradiation
(Readout: Figure 8D)


C
Pregated on donor Lineage-
PBS
G-CSF
CXCR4 antagonist
c-Mpl agonist
Flt3L
>5 5 4 3 2 1 0
c-Kit
Sca-1
CFSE

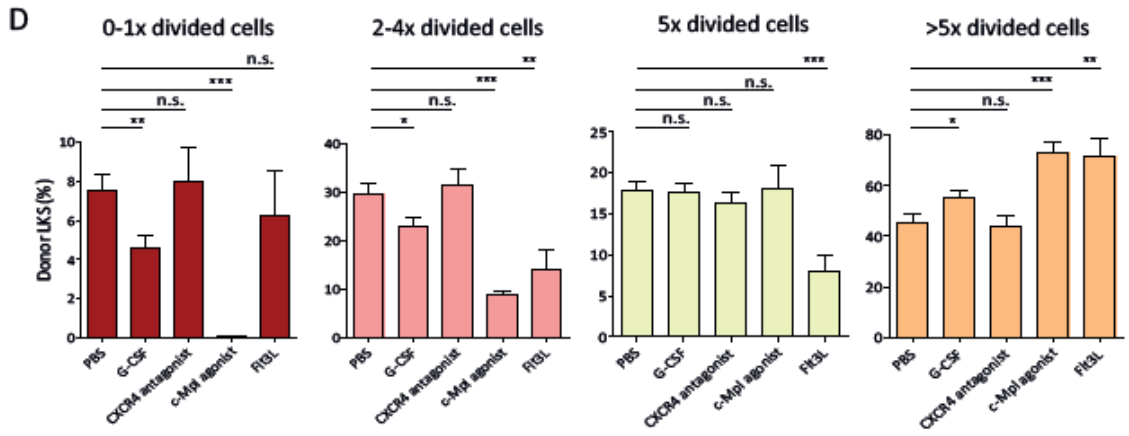

D
0-1x divided cells
2-4x divided cells
5x divided cells
>5x divided cells
Donor LKS (%)
n.s.
***
**
*
PBS
G-CSF
CXCR4 antagonist
c-Mpl agonist
Flt3L

Figure 7. Receptor expression and proliferation of LKS upon extrinsic stimulation. (A) Quantitative PCR analysis on mRNA expression of the indicated genes in FACS-sorted BM HSCs (LKS $Flt3^{-}CD34^{-}CD48^{-}CD150^{+}$), MPPs (LKS $Flt3^{-}CD34^{+}$), GMPs (LK $Sca1^{-}FcgR^{+}CD34^{+}$) and granulocytes ($Mac1^{+}Gr1^{+}$), as well as spleen T ($CD3^{+}$), B ($CD19^{+}$) and dendritic cells ($CD11c^{+}MHCII^{+}$). Relative expression of the indicated genes to the expression of Actb is shown in respective cell populations (mean ± SEM), data obtained from 3 independent experiments, n=4. (B) Experimental scheme: steady-state, non-irradiated mice were transplanted with 0.8-1.1 x 105 CFSE-labeled LKS cells and one week post transplantation, injected over one week as indicated in methods. One week after the final injection and 3 weeks after transplantation of CFSE-labeled LKS, mice were analyzed and cells were subsequently sorted for transplantation as indicated. (C) Representative dot plot analysis of lineage-depleted BM cells pre-gated on donor Lin^{-} (upper) or donor $Lin^{-}c\text{-}Kit^{+}$ cells (lower). (D) Percentage of donor LKS in subsequent divisional groups. Graphs show mean ± SEM (n=6-24 mice from 3 to 14 independent experiments). Data analyzed with paired, two tailed T-test. ***p<0.001, **p<0.01, *p<0.05, n.s. not significant.

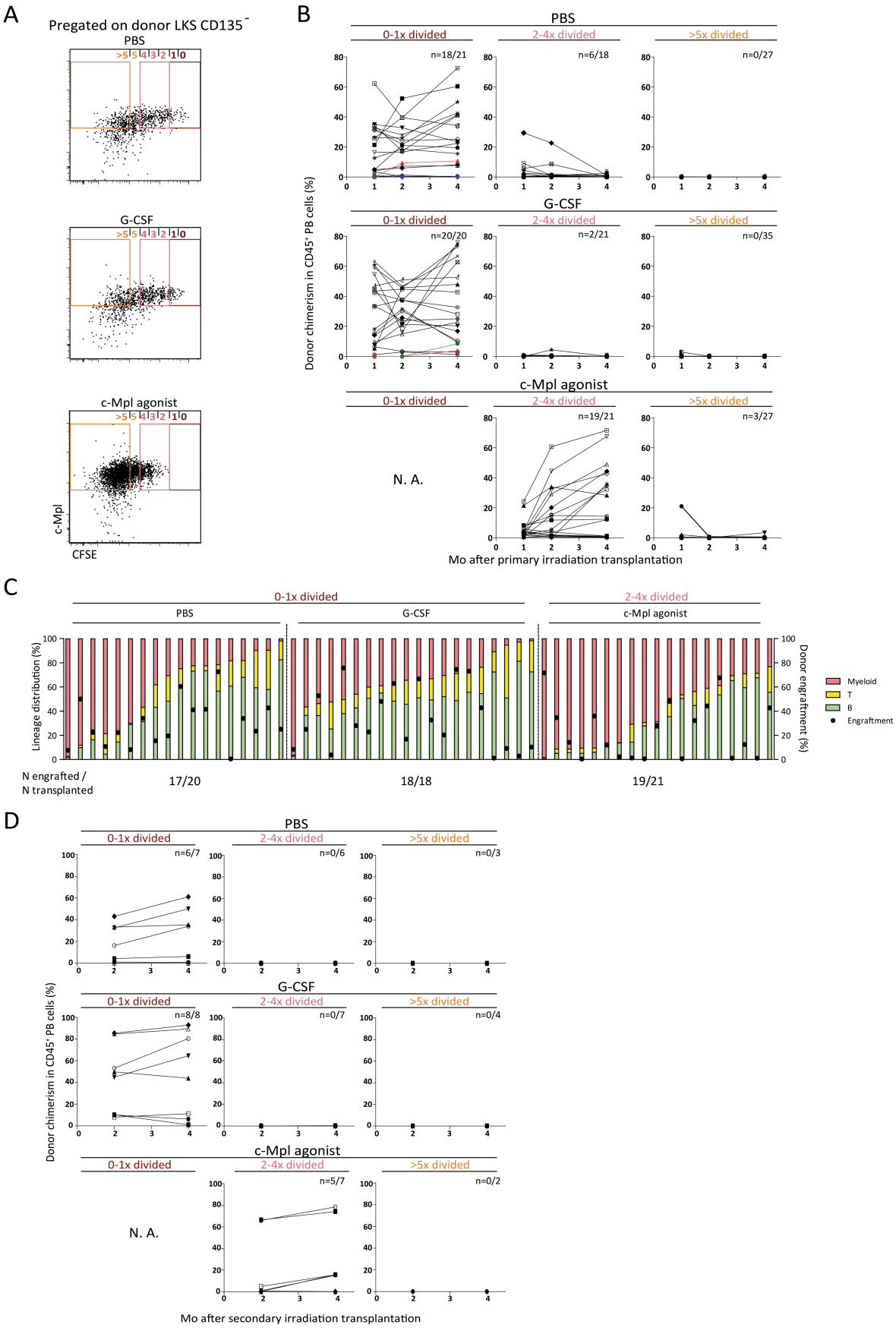

A
Pregated on donor LKS CD135-
PBS
G-CSF
c-Mpl agonist
>5 5 4 3 2 1 0
c-Mpl
CFSE
B
PBS
G-CSF
c-Mpl agonist
0-1x divided
2-4x divided
>5x divided
n=18/21
n=6/18
n=0/27
n=20/20
n=2/21
n=0/35
n=19/21
n=3/27
N. A.
Donor chimerism in CD45+ PB cells (%)
Mo after primary irradiation transplantation
C
0-1x divided
2-4x divided
PBS
G-CSF
c-Mpl agonist
Lineage distribution (%)
Donor engraftment (%)
Myeloid
T
B
Engraftment
N engrafted /
N transplanted
17/20
18/18
19/21
D
PBS
G-CSF
c-Mpl agonist
0-1x divided
2-4x divided
>5x divided
n=6/7
n=0/6
n=0/3
n=8/8
n=0/7
n=0/4
n=5/7
n=0/2
N. A.
Donor chimerism in CD45+ PB cells (%)
Mo after secondary irradiation transplantation

Figure 8. cMpl-agonist treatment induces HSC division and self-renewal. (A) Representative BM FACS plots (pre-gated on donor LKS $Flt3^-$) from animals transplanted three weeks prior with CFSE-labeled LKS cells and subsequently treated with PBS, G-CSF or cMpl agonist as in Figure 7B-D. Sorting gates are indicated. (B) Donor chimerism in PB over 4 month of recipients of cells sorted from 0-1x-, 2-4x- and >5x-divided cells from PBS, G-CSF and cMpl-agonist-treated animals as indicated. Graphs show individual donor chimerism from animals transplanted with 7 cells (blue line, n=1), 12 cells (red lines, n=2), 17 cells (green line, n=1) and 20-22 cells (black lines). Number of animals engrafted (positive cut-off >0.4%)/ number of animals transplanted is indicated on each graph. (C) Donor cell engraftment (cut-off >0.4%) and lineage distribution (myeloid lineage, T cells and B cells) in recipient mice PB at month four. (D) Donor chimerism in PB over 4 months in recipients of WBM from animals in B and C. Number of engrafted versus transplanted animals (positive cut-off >0.4%) is shown in each graph.

ACKNOWLEDGEMENTS

This work was supported by the Swiss National Science Foundation (310030_146528), the Promedica Foundation (Chur, Switzerland) to M.G.M., and in part by the Japanese Society for the Promotion of Science KAKENHI (15H01519) to H.T.

SUPPLEMENTAL DATA

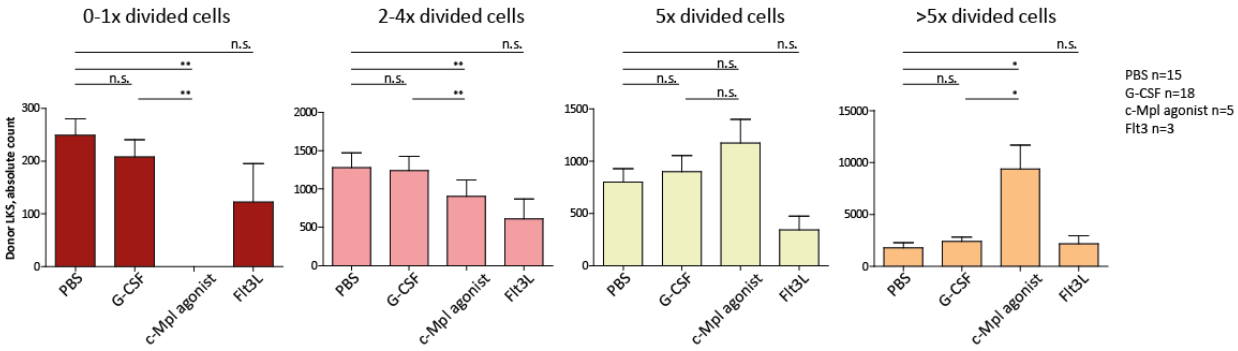
Supplemental Table 1.

Average numbers of donor LKS Flt3$^-$cMpl$^+$ cells sorted from PBS, G-CSF and cMpl agonist treated animals and estimated frequency of multi-lineage repopulating HSC within this populations

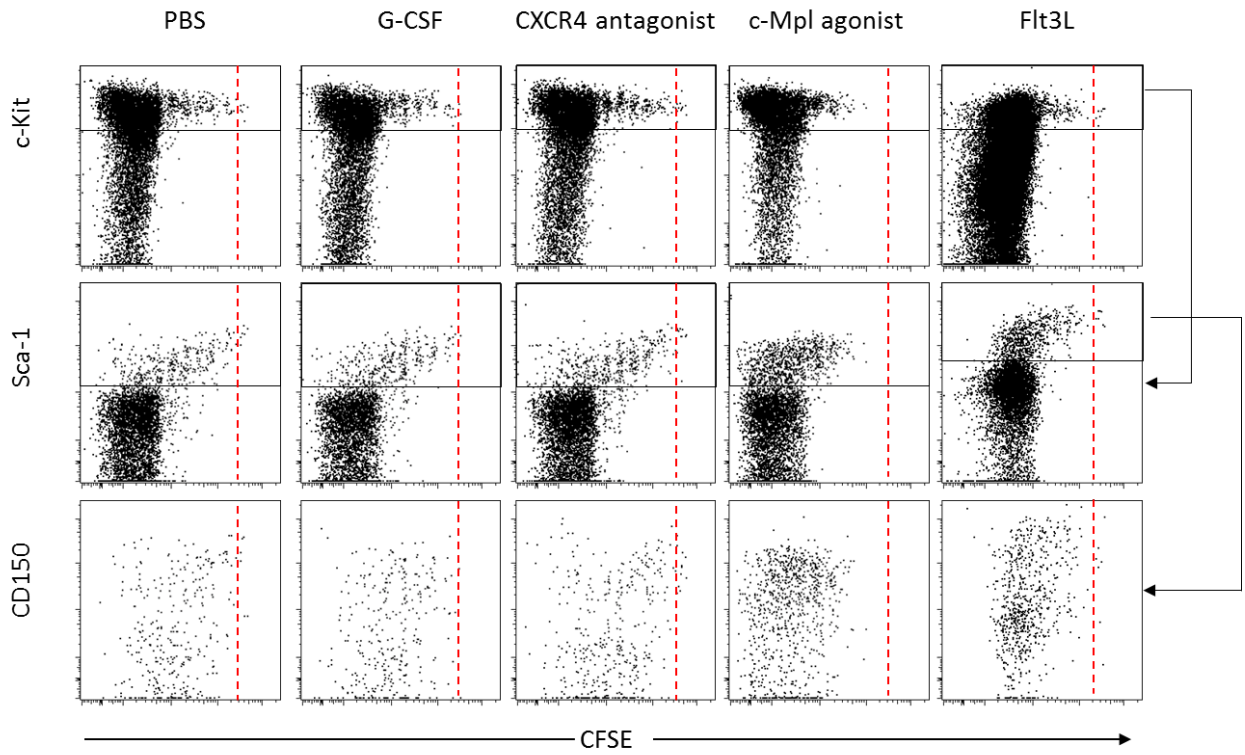
Experiment	Treatment	N of animals pooled and analyzed	Average numbers of CD45.2$^+$ LKS Flt3$^-$cMpl$^+$ cells sorted per single animal			
			0-1x divided cells	2-4x divided cells	>5x divided cells	total
1	PBS	4	11.3	31.5	42.8	85.5
	TPO	4	0.3	77.8	895.0	973.0
2	PBS	1	12.0	34.0	55.0	101.0
	TPO	1	0	13.0	390.0	403.0
3	PBS	4	8.0	27.5	13.5	49.0
	G-CSF	4	16.5	55.0	52.5	124.0
	TPO	1	0	105.0	400.0	505.0
4	PBS	3	37.7	56.3	26.0	120.0
	G-CSF	3	34.0	116.7	75.3	226.0
	TPO	1	0	160.0	1488.0	1648.0
5	PBS	4	26.8	52.8	29.0	108.5
	G-CSF	6	19.5	83.3	275.7	378.5
	TPO	1	0	30.0	480.0	510.0
6	PBS	3	56.0	165.3	154.3	375.7
	G-CSF	5	73.6	338.2	203.4	615.2
7	PBS	3	18.0	50.7	66.7	135.3
	G-CSF	6	15.8	66.5	68.3	150.7
8	PBS	2	24.5	63.0	46.5	134.0
	G-CSF	3	16.7	73.7	100.3	190.7
Mean ± SEM	PBS		24.3±5.7	60.1±15.7	54.2±15.5	138.6±35.3
	G-CSF		29.4±9.3	122.2±44.0	129.3±36.6	280.3±76.2
	TPO		0.1±0.1**	77.2±26.4	730.6±210.8**	807.8±232.1**
Estimated HSC frequency	PBS		1/11.5	1/53.3	0	-
	G-CSF		n.a.	1/210.3	0	-
	TPO		n.a.	1/9.4	1/178.8	-
Estimated average N of HSCs sorted per animal	PBS		2.1	1.1	0	3.22
	G-CSF		n.a.	0.6	0	0.6
	TPO		n.a.	8.2	4.1	12.3

Table depicts the average numbers of donor–type (CD45.2$^+$) LKS Flt3$^-$cMpl$^+$ cells per animal that were actually sorted from the indicated number of pooled animals (Mean ± SEM). The number of donor HSCs was calculated per single animal based on cell dose and on frequency of multi-lineage repopulating cells estimated from the engrafting efficiency in serial transplantation as depicted in Fig. 2, b and c. n.a., not applicable., ** $p<0.01$, compared to PBS control.

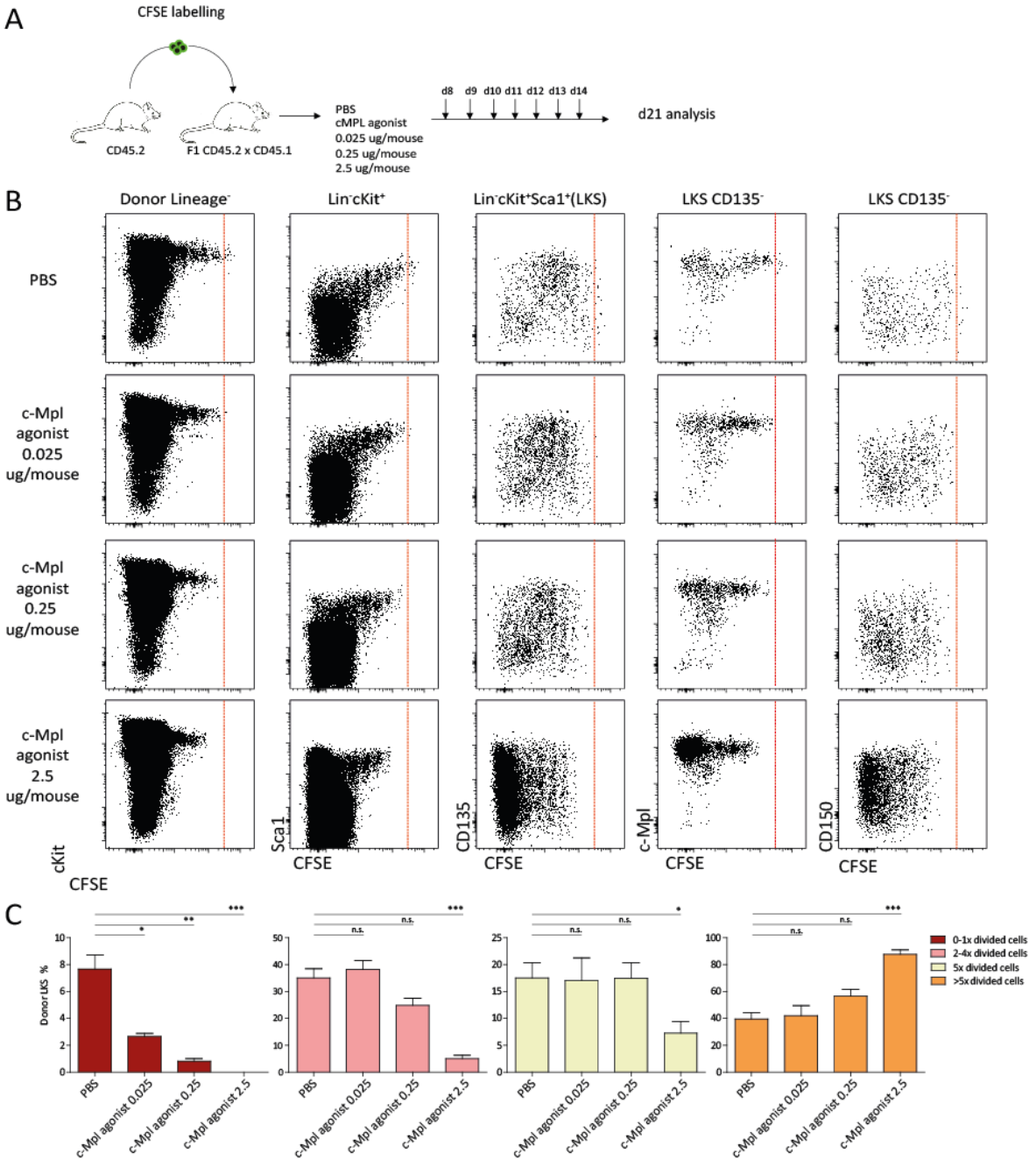
SUPPLEMENTAL FIGURES



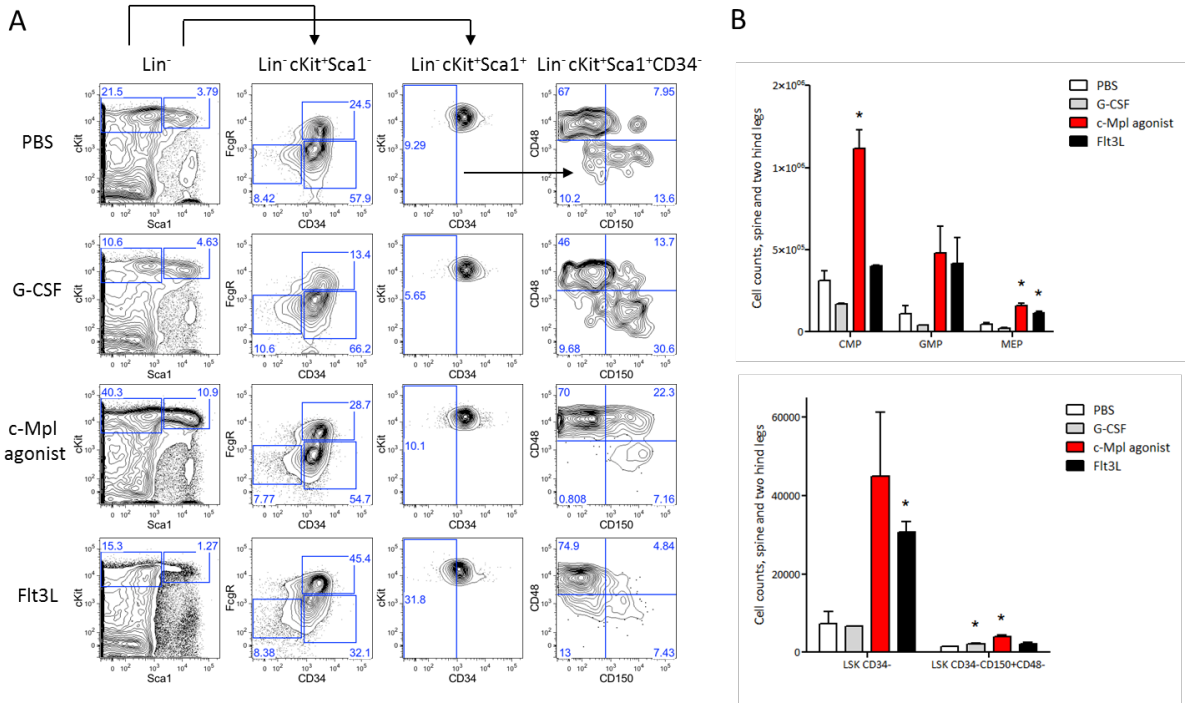
Supplemental Figure 1. Proliferation of LKS upon cMpl, but not G-CSF and Flt3 activation. Absolute numbers of donor LKS were calculated in the data from the experiments shown in Figure 7B and 7C. * $p<0.05$, ** $p<0.01$ compared to PBS control, n.s. non-significant.



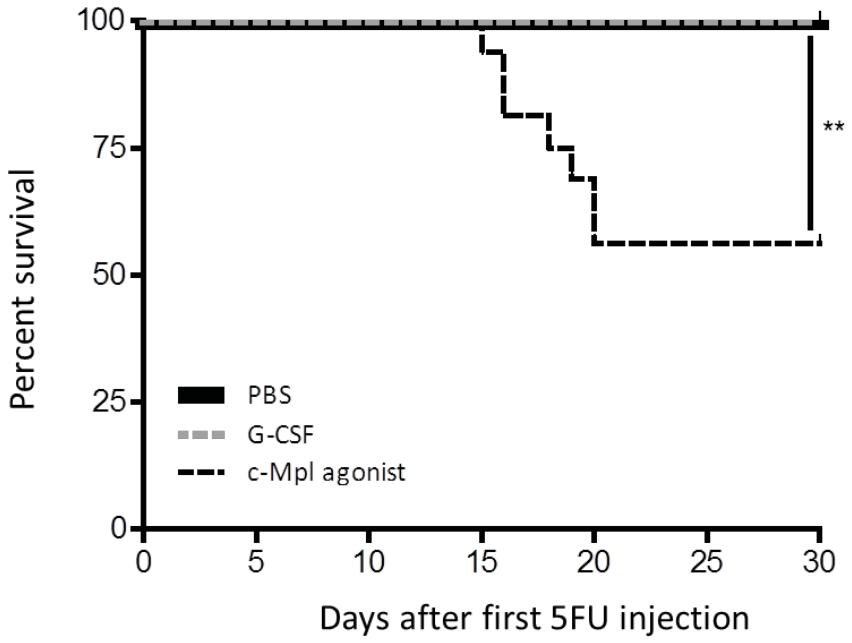
Supplemental Figure 2. Proliferation of CD150^{+} LKS upon extrinsic stimulation. Representative dot plot analysis of c-Kit, Sca-1 and CD150 expression on lineage-depleted BM cells pre-gated on donor Lin^{-}, or donor Lin^{-}c-Kit^{+} cells, or donor Lin^{-}c-Kit^{+}Sca-1^{+}. The experiment was done as experiments in Figure 7 but with additional CD150 staining. Dashed red line represents 0 divisional gate according to CFSE intensity in non-divided CD4^{+}CD62L^{+} T cells.



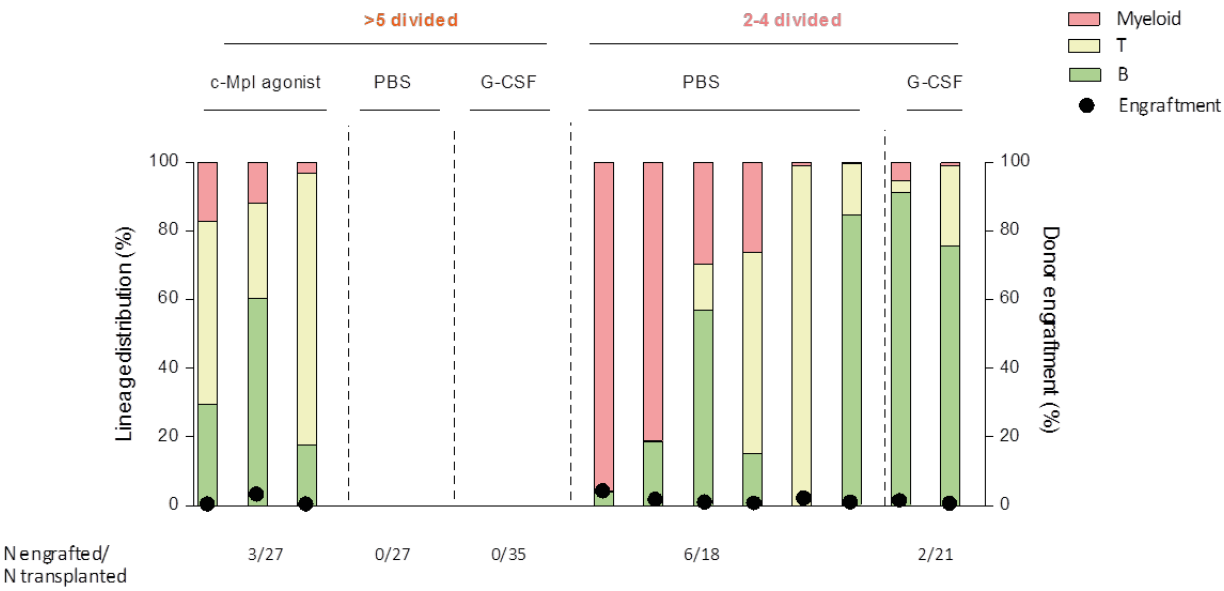
Supplemental Figure 3. **Dose dependent effect of cMpl agonist treatment.** (A) Experimental Scheme: CD45.1/2^{+} mice were transplanted with 1.0x10^{5} CFSE-labeled CD45.2^{+} LKS cells. 1 week later, they were i.p. injected daily for 7 days with PBS, 0.025 µg, 0.25 µg or 2.5 µg cMpl agonist per mouse. BM was analyzed 1 week after the final injection and 3 weeks after CFSE chasing. (B) Representative dot plot of BM cells pre-gated on donor Lin^{-}, donor Lin^{-}c-Kit^{+} cells, donor LKS or LKS CD135- cells. Dashed red line represents 0-divisional gate according to CFSE intensity in non-divided CD4^{+}CD62L^{+} T cells. (C) Percentage of donor LKS in subsequent divisional groups. Graphs show mean ± SEM (n=3-5 mice from 3 independent experiments). Data analyzed with paired, two tailed T-test. ***p<0.001, **p<0.01, *p<0.05, n.s. not significant.



Supplemental Figure 4. Short term stimulation of mice with cytokines. Mice were stimulated with cytokines according to the scheme of injections in Figure 7B. One hour after the last injection BM was isolated and analyzed for HSC and progenitor cell populations. (A) Representative FACS plots. (B) Absolute cell counts of HSC and progenitors. n=2 per group, * $p<0.05$ compared to PBS control, otherwise non-significant.



Supplemental Figure 5. **In vivo cMpl agonist treatment induces increased toxicity of 5FU.** Survival of mice following either seven times of PBS (day -7 to day -1, n=16), five times of G-CSF (day -5 to day -1, n=14) or seven times of cMpl agonist injections (day -7 to day -1, n=16) and each subsequent treatment with 150 mg/kg 5-FU at day 0 and 7. ** $p<0.01$, cMpl agonist-treated mice compared to PBS control mice.



Supplemental Figure 6. Lineage distribution and engraftment of 2-4 divided cells from PBS and G-CSF, and >5 divided cells from cMpl agonist treated animals upon transplantation. Lineage distribution and engraftment of donor derived cells in individual recipients after primary lethal irradiation transplantation (>0.4%). Donor cell lineage distribution (myeloid lineage, T cells and B cells, color coded as indicated on the graph) in total CD45 cells in peripheral blood at 4 months after lethal irradiation and transplantation. Dots indicate donor cell engraftment level in individual mouse.

RESEARCH ARTICLE 2

(Draft in preparation)

AGEING ASSOCIATED INTRINSIC AND EXTRINSIC FACTORS CONTROL HEMATOPOIETIC STEM CELL BEHAVIOR

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*Ramin Radpour and Steffen Boettcher performed transcriptomics analysis, rest was performed by Larisa Kovtonyuk.

ABSTRACT

Life-long self-renewing hematopoietic stem cells (HSCs) replenish mature blood cells each day. Upon aging, HSCs reduce their self-renewal capacity, skew towards myeloid differentiation and show inefficient bone marrow (BM)–homing ability. Recently we have demonstrated that at any given time, long-term HSCs consist of actively cycling and dormant pools. Here we demonstrate that HSC behavior during ageing is controlled in an intrinsic fashion, as cells with high proliferative history increase their quiescence. When challenged with proliferative stimuli, aged HSCs display a slower response compared to young HSCs. Furthermore, we also demonstrate that it is possible to reverse the myeloid bias of naturally or experimentally aged HSCs via extrinsic factors.

MATERIALS AND METHODS

Mice

C57BL/6J (CD45.2$^+$) and B6.SJL (CD45.1$^+$) mice were obtained from Jackson Laboratories, B6.JRj (CD45.2$^+$) mice were obtained from Janvier and CD45.1/2$^+$ mice were generated by intercrossing C57BL/6J (CD45.2$^+$) with B6.SJL (CD45.1$^+$) or B6.JRj (CD45.2$^+$) with B6.SJL (CD45.1$^+$). IL1RI KO mice were provided by Manfred Kopf, Institute of Molecular Health Sciences, ETHZ. Mice were maintained in the University Hospital Zurich animal facility according to the guidelines of the Swiss Federal Veterinary Office, and all experiments were approved by the Veterinärmt of Kanton Zurich, Zurich, Switzerland (54/2010, 55/2010, 186/2013 license number). Mice aged between 7-14 weeks were used for experiments and considered as young adult mice, mice aged 2 years and more were used for ageing experiments and considered as aged mice.

Peripheral blood sampling, cell isolation,

BM cells were harvested from hip bones, femurs, tibias and spine. Single cell suspensions were generated and red blood cells were lysed with red blood cell (ACK) lysis buffer (150 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA). Cell debris was removed with 70-µm filters (BD).

For peripheral blood cell analysis 75ul blood was drawn from sublingual or retro-orbital veins. Red blood cells were lysed with 800ul of ACK lysis buffer.

For serum isolation, blood (500-700ul) was collected post-mortem via cardiac puncture, left at room temperature for 10 minutes and then centrifuged at 10.000 rpm (Hettich Laborapparate Zentrifugen, Mikro 220R) for 10 minutes. Cell free supernatant was collected and stored at -80 C^o.

FACS analysis and sorting

For LKS sorting, cells were stained with biotin–conjugated antibodies against the lineage antigens B220 (RA3-6B2), CD3ε (145-2C11), Ter119 (Ter119), NK1.1 (PK136), CD11b (M1/70), CD4 (RM4-5), CD8α (53-6.7), IL-7Rα (A7R34) and Gr-1 (RB6-8C5) and immunomagnetically enriched for lineage-negative cells using streptavidin microbeads (Miltenyi Biotec). The lineage-depleted cells (Lin$^-$) were subsequently stained with Pacific Blue conjugated streptavidin, PE-Cy5–conjugated antibody to c-Kit (2B8) and APC-Cy7-

conjugated antibody to Sca-1 (D7). LKS were sorted using a FACSAria III (BD Biosciences). Naive $CD4^+CD62L^+$ T cells were enriched from spleen cells with biotin–conjugated anti-CD4 antibody (GK1.5) and streptavidin microbeads, and then stained with PE-Cy5 conjugated streptavidin and PE-conjugated anti-CD62L antibody (MEL-14). $CD4^+CD62L^+$ T cells were sorted on a FACSAria III.

For 3 week or 8 week CFSE chasing analysis and sorting, cells were enriched for lineage-negative cells: WBM cells were stained with Biotin conjugated antibodies against lineage antigens listed above and subsequently stained with Streptavidin Microbeads (Miltenyi Biotec) and enriched on MACS columns. Lineage depleted cells were then stained with PE-Cy5 cKit (2B8), APC-Cy7 Sca1 (D7), SA-Pacific Blue, PE CD150 (TC15-12F12.2), APC CD45.1 (A20), PE-Cy7 CD45.2 (104). The non-divisional (0-division) cell gate was set according to the CFSE intensity in the donor $CD4^+CD62L^+$ cells. For this, spleen cells of primary recipients of $CD4^+CD62L^+$ cells were mashed and red blood cells were lysed with ACK buffer.Cells were stained with PE-Cy5 CD4 (RM4-5), PE CD62L (MEL-14), PE-Cy7 CD45.1 (A20), APC CD45.2 (104). Single cell division was determined as described (Takizawa et al. 2011). Donor LKS from 0x divided cells and >5x divided cells were then sorted and transplanted into lethally irradiated (9.5cGy) $CD45.1^+$mice.

Total BM of secondary and tertiary recipients was harvested as above, stained with biotinylated lineage markers (as indicated above), and antibodies conjugated with fluorochromes against the following antigens: PE-Cy5 cKit(2B8), APC-Cy7 Sca1 (D7), PE CD150 (TC15-12F12.2), Alexa Fluor 660 CD34 (RAM34), PE-Cy7 CD45.1 (A20), FITC CD45.2 (104). Peripheral blood analysis was performed as previously described (Takizawa et al. 2011). Briefly peripheral blood was harvested, depleted from erythrocytes, and stained with antibodies against FITC-CD45.1 (A20), PE-CD45.2 (104), PE-Cy5-CD19 (1D3), PE-Cy7-CD3ε (2C11), APC-Gr-1 (RB6-8C5) and eFluor780-CD11b (M1/70).

For long-term cMpl agonist treatment, BM stroma cell analysis cells were obtained from 2 tibias, 1 femur, 2 hip bones. Bones were flushed, and the flushed bones were minced with scissors. Then, the bone fragments were incubated with Digestion medium containing DMEM (Gibco), 10% FBS (Gibco), 10mM HEPES (ThermoFisher), 0.02% DNAse (Roche) and 0.4% type II collagenase (Worthington Biochemical, Lakewood, NJ) for 45 min at 37 °C. The cell suspensions were filtered with a 70-µm cell filter. Cells were stained with antibodies conjugated with fluorochromes against the following antigens: BV510-Ter119 (TER119),

BV510-CD45 (30-F11), APC-Cy7 Sca1 (D7), PE-Cy7- CD31 (390), APC-CD140b (APB5), BV421-CD105 (MJ7/18) and biotinilated-CD51 (RMV7), followed by SA-FITC (eBioscience) staining.

Before analysis, cells were resuspended in PBS containing 2% FBS, 0.002M EDTA and 2 µg/ml Hoechst 33342 (Invitrogen) or PBS containing 2%FBS, 0.002M EDTA and 2 µg/ml Propidium Iodide (Invitrogen). All antibodies were obtained from BioLegend, eBioscience or Life technologies.

CFSE labelling and Cytokine injections

LKS cells were FAC-sorted and labelled with 2µM 5-(and-6)-Carboxyfluorescein Diacetate, Succinimidyl Ester (5(6)-CFDA, SE; CFSE) (Invitrogen) as previously described (Takizawa et al. 2011). Non-irradiated mice were i.v. transplanted with 0.8-1.1x10^5 LKS cells per mouse. One week after transplantation mice were injected i.p. with PBS (x7injections), 2.5 ug/mouse cMPL agonist (x7injections, Nplate, Amgen), 1 ug/mouse of IL-1α (R&D systems) or IL-1β (BioLegend) and analyzed 1 week after the final injection and 3 weeks after divisional tracking.

Long-term cytokine or cytokine antagonist treatment

For long-term treatment mice were injected weekly with with cMPL agonist (Nplate, 2,5 ug/mouse) over 2 or 6 month. Mice were used for further experiments at 2 or 4 month after the last injection.

Blood counts on FACS Advia

To control platelet counts post cMpl agonist long-term treatment blood was drawn via sublingual bleeding and stored in EDTA coated tubes. For analysis, blood was diluted in a 1:2 ratio. Animals were analyzed when their platelet counts were not more than 1.5-2 times more in the cMpl agonist treated animals compared to PBS treated animals.

Cytokine array

Cytokine Antibody Array (array numbers 3, 4 and 5) was purchased from RayBiotech and performed according to manufacturer's manual.

ELISA

ELISA kits for mouse thrombopoietin, IL-1alpha, IL-1beta, MIP-2 were purchased from R&D systems, and performed according to manufacturer's manual.

Transplantation

All cells were transplanted i.v. and resuspended in 200µl of PBS per mouse. For comparison of young and aged HSC proliferative rate after 8 weeks of CFSE chasing, LKS cells were FAC-sorted according to the divisional groups (0-divided and >5 divided) and mixed with $CD45.1^+$ whole BM at dose (per mouse) of 20-22 of 0x divided and 150-250 >5 divided $CD45.2^+$ LKS plus $3x10^5$ $CD45.1^+$ WBM cells and transplanted into lethally irradiated (9.5 cGy) $CD45.1^+$ B6.SJL or $CD45.1/2^+$ B6.SJLxC57BL/6 mice.

Statistics

All statistical analysis was done either in Excel or in GraphPad Prism. Error bars represent ±SEM. Data sets were compared either by paired, two-tailed Student T-test or two-way Anova test (as indicated in figure legends). P-value was considered significant at values $p<0.05$.

PRELIMINARY RESULTS

Intrinsic and extrinsic control of the HSC ageing phenotype

In order to address the question of how divisional dynamics of HSCs change with ageing-associated cell-intrinsic and –extrinsic factors, I employed single HSC divisional tracking over time by using CFSE-dilution. CFSE-labeled young (8-12 week old) or old (>2 years old) LKS that contain 3% of functional HSCs was transferred into non-irradiated steady-state young (8-12 wk old) or old (>2 years old old) recipients (Figure 11).

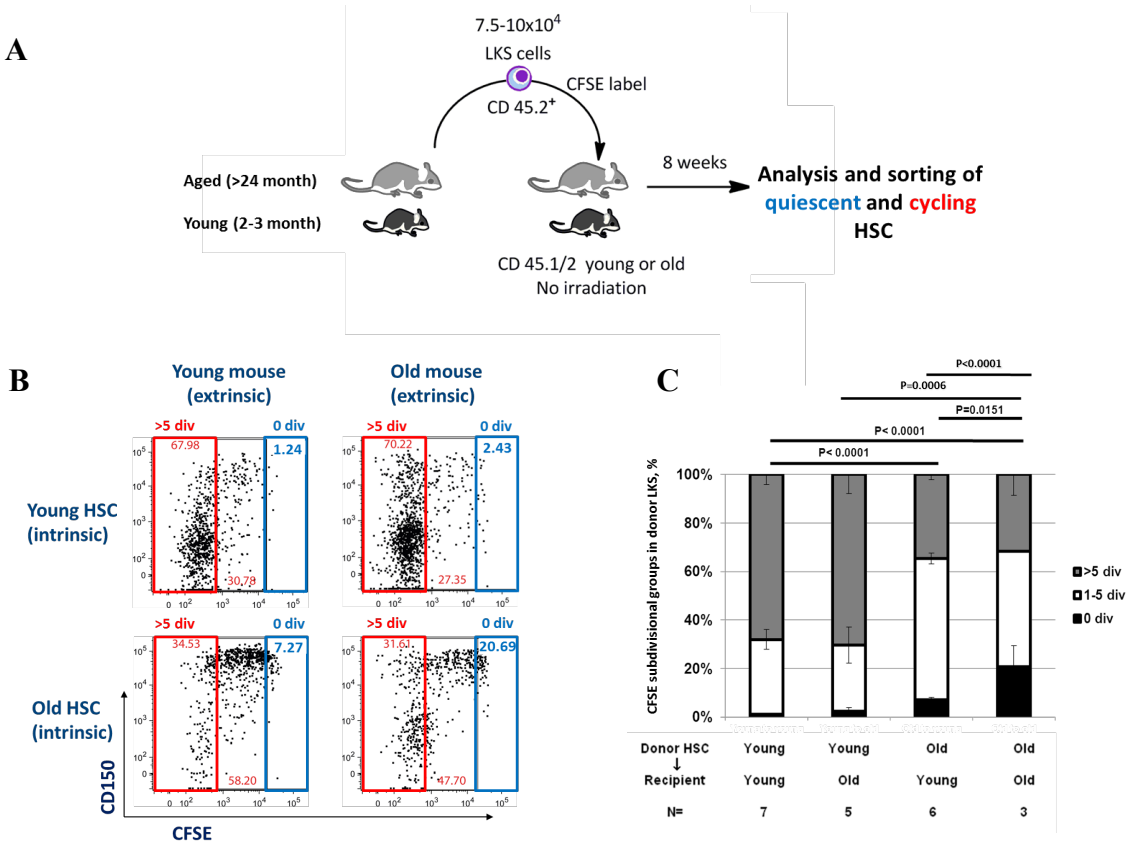

Figure 11: **Analysis of cell cycle rate of HSCs** A. Experimental scheme of CFSE-based cell cycle tracking and serial transplantation for assessment of HSC function. B. Representative FACS profile of divisional history of young vs. old LKS transplanted into young vs. old environment. C. Percentage of donor LKS in subsequent divisional groups: non-divided cells (0 div., black), slow- (1-5 div., white) and fast cycling cells (>5 div., grey). Graph shows mean ± SEM (n=3-7 mice from three independent experiments). P-value indicated was assessed by T-test on 0 divided fraction.

Eight weeks after steady-state tracking, young LKS proliferated faster than old LKS in both the young and old BM environment ($p<0.0001$). In contrast, both young and old LKS appear to be more dormant in an old environment (Figure 11). These suggest that a strong intrinsic drive towards quiescence imprints on HSCs with high proliferative history, whereas extrinsic signals accelerate cell turnover of aged HSCs ($p=0.0151$), and does not affect high proliferative capacity of young HSCs.

To functionally test HSCs with distinct cell division kinetics, dormant (0-divided at 8 weeks) or cycling LKS (>5-divided at 8 weeks) ($CD45.2^+$) were isolated and transplanted into lethally irradiated secondary recipient mice ($CD45.1^+$ or $CD45.2^+/CD45.1^+$) along with a rescue dose of total BM cells from another donor ($CD45.1^+$). Young HSC, irrespective of environment or cycling activity, demonstrated balanced lineage repopulation. Dormant old HSCs, independent of environment, favor myelopoiesis (Figure 12). In contrast, cycling old HSCs that had been exposed to a young environment showed balanced lineage differentiation, as do young HSCs. This indicates that the ageing-associated lineage-skewing program can be reversed through cell division that is induced in a young environment. A similar behavior as in old HSCs was observed in HSCs with extensive divisions during serial transplantation. These data suggest that during ageing, HSC acquire intrinsic changes as increased quiescence and a myeloid biased program, which both can be modulated by extrinsic young factors that drive HSCs to proliferate. It has been previously shown that the lineage choice of HSCs is controlled via epigenetic mechanisms (Challen et al. 2011). Thus, it will be interesting to understand how extrinsic factors affect the epigenetic nature of HSC ageing.

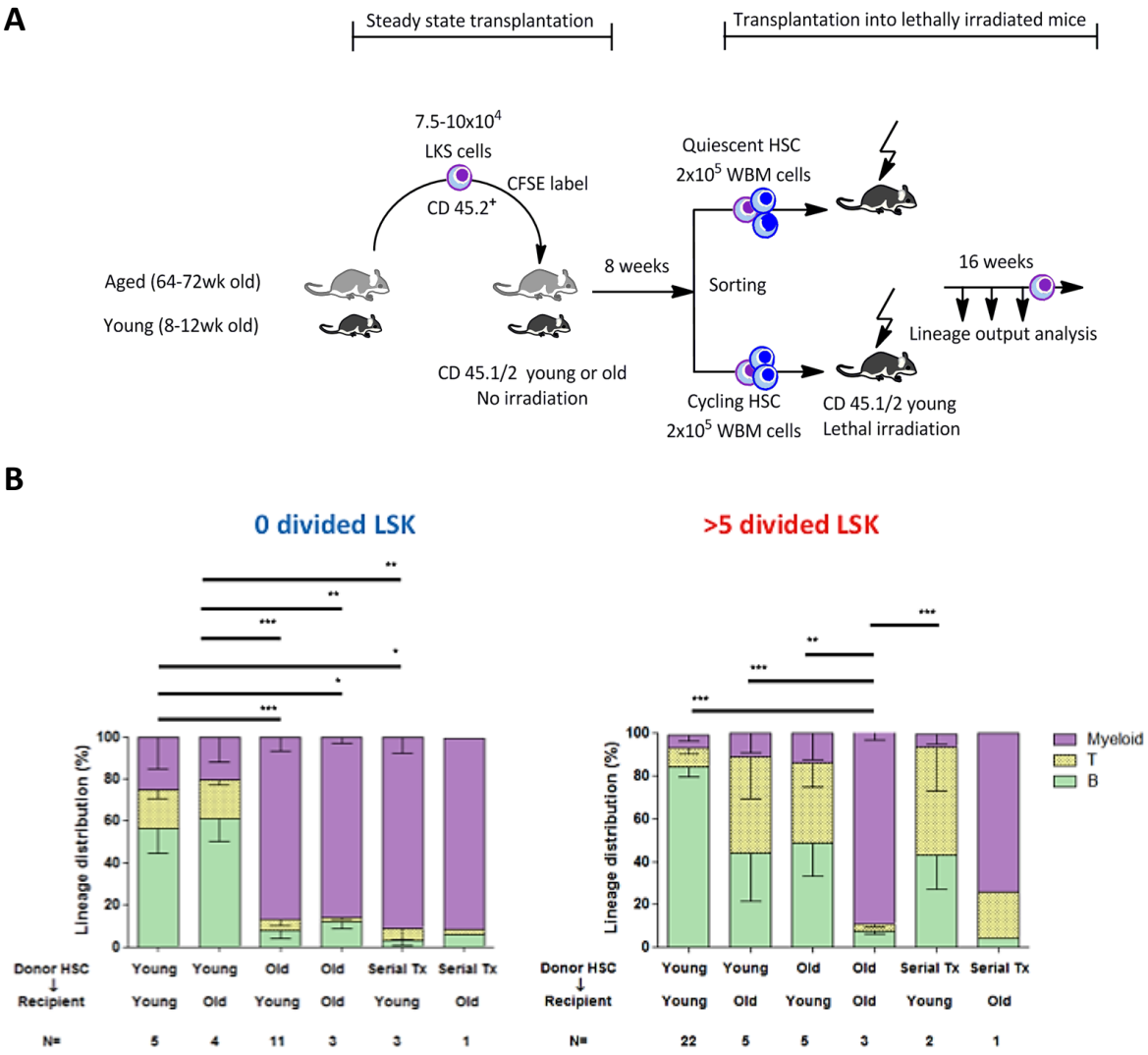

Figure 12: **Lineage outcome of quiescent and cycling HSCs upon lethal irradiation transplantation.** A. Experimental scheme. B. Lineage contribution of quiescent and cycling donor cells in peripheral blood 16 weeks after transplantation. Statistical analysis on myeloid lineage was done by t-test, P value is indicated above the respective graph, *p<0.05, **p<0.01, ***p<0.001 otherwise n.s. Number of experimental animals is indicated under the graph. Graph shows mean ± SEM (n=1-22 mice from three independent experiments).

HSCs with high proliferative history partially mimic an ageing phenotype

To estimate the long-term effect of cMpl agonist treatment on the early HSPC compartment, mice were treated over 2 month weekly with cMpl agonist. One month later bone marrow was analyzed for the early HSPC compartment. cMpl agonist treatment increased LKS $CD41^{-}CD48^{-}CD150^{+}$ cells by almost 3-fold (Figure 13 A and B). In contrast, phenotypic MPPs and HPCs (LKS $CD41^{-}CD48^{-}CD150^{-}$ and LKS $CD41^{+}CD48^{+}$) were not significantly increased (Figure 13 B). To estimate cycling kinetics of HSCs subjected to long treatment with cMpl agonist, LKS cells from mice treated for 2 month with cMpl agonist (cMpl agonist pre-treated cells) or PBS were further sorted, labeled with CFSE and transplanted into steady-state F1 recipients. BM analysis after three weeks demonstrated that cells with higher proliferative history due to cMpl pre-treatment, are able to form quiescent HSC populations (i.e. cells are present in non-divisional gate – marked with red dashed line in Figure 13C). This indicates that the proliferative effect of cMpl agonist is terminated. Moreover, cMpl agonist pre-treated cells increase their quiescence in the LKS $CD48^{-}CD150^{+}$ phenotypic HSC compartment (Figure 13 C and D), as compared to PBS pre-treated donor cells.

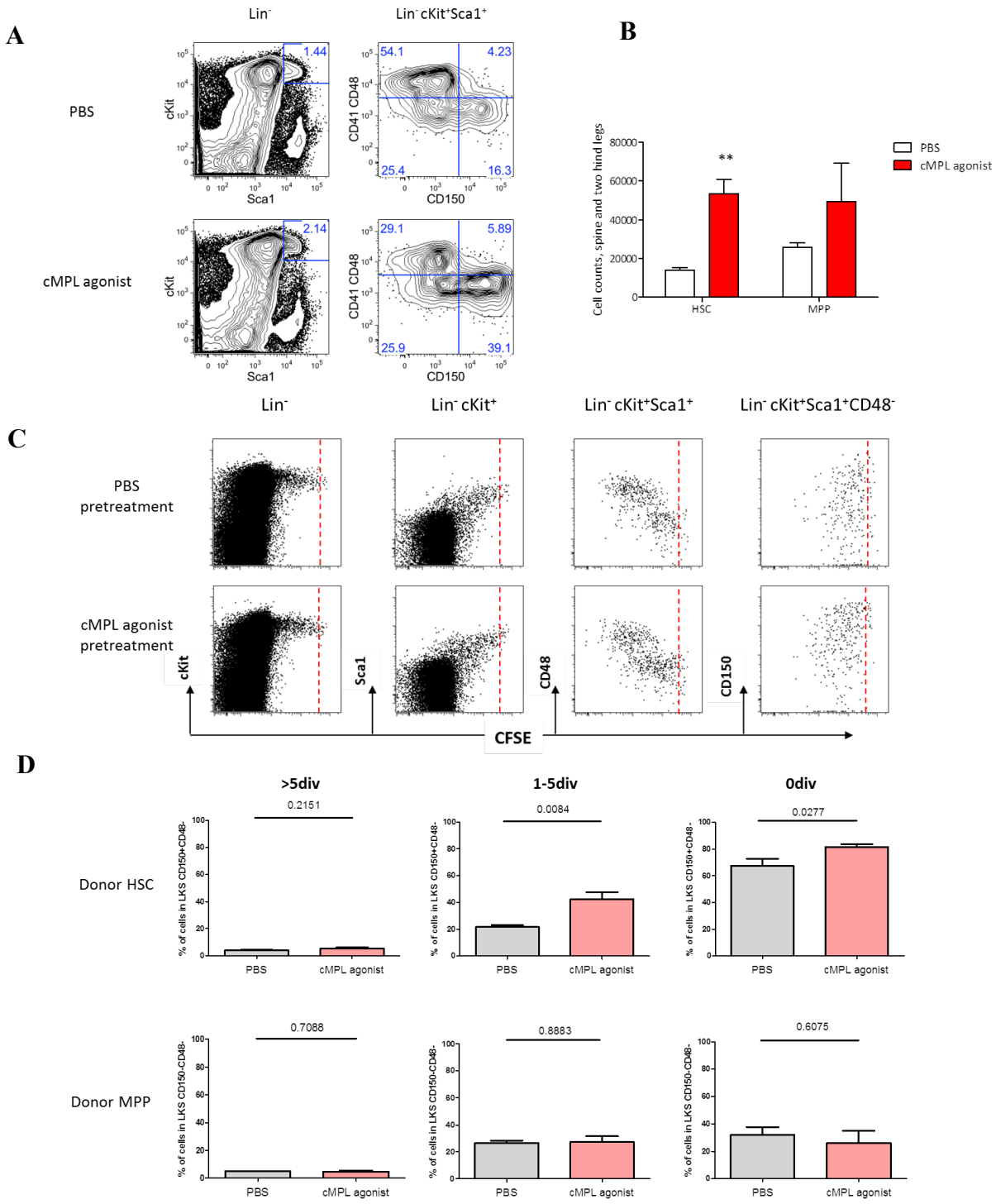

Figure 13: **Long term stimulation of mice with cMPL agonist. A.** Representative FACS plots. **B**. Absolute cell counts per spine, 2 hind legs. HSC = LSK $CD150^{+}CD41^{-}CD48^{-}$, MPP = LSK $CD150^{-}CD41^{-}CD48^{-}$, n=4 per condition, data obtained from 2 independent experiments. Graph shows mean±SEM, data analyzed with paired, two-tailed T-test, ** $p<0.01$. **C.** CFSE chasing 3 weeks after transplantation of donor LKS pretreated with PBS or cMPL agonist, representative FACS plots, dashed red line indicates 0 divisional gate. **D.** Donor LKS cells 3 weeks after CFSE chasing were divided in 4 subpopulations according to CD150 and CD48 expression, LKS $CD150^{+}CD48^{-}$ cells are referred as donor HSC, LKS $CD150^{-}CD48^{-}$ cells are referred as donor MPP. Data obtained from 2 independent experiments, analyzed with paired, two-tailed T-test, P value indicated on top of the graph.

To understand whether HSC expansion can be further improved and up to what extent it can be reached, we investigated how the cMpl agonist long-term (over 6 month) treatment affects the early HSPC compartment. Mice were treated over 6 month weekly with cMpl agonist (2.5 µg per mouse) or PBS. Four month later peripheral blood and bone marrow compartments were analyzed and compared with respective tissues in aged mice. As expected, myeloid lineage is prevalent in peripheral blood of aged mice (>50% of total CD45 positive cells were myeloid) compared to PBS-treated mice (~50% myeloid and ~50% lymphoid cells, indicating balanced lineage distribution). Interestingly, cMpl agonist treatment lead to a slight increase in the ratio of myeloid cells ($CD45^+CD3\varepsilon^-CD19^-Gr1^+Mac1^+$ cells in PBMC) over lymphoid cells ($CD45^+CD3\varepsilon^+$ and $CD45^+CD19^+$) in peripheral blood (Figure 14).

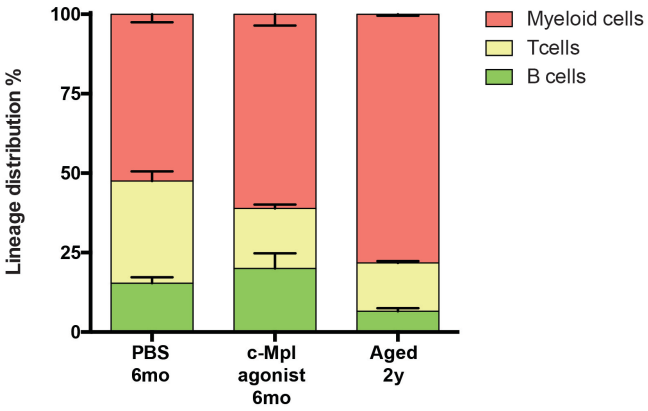

Figure 14: **Lineage distribution in peripheral blood $CD45^+$ cells.** FACS analysis of blood composition, relative % of total $CD45^+$ cells: red color represents myeloid lineage ($CD45^+CD3^-CD19^-$), yellow color T cells ($CD45^+CD3^+CD19^-$), green B cells ($CD45^+CD3^-CD19^+$), graph shows mean and S.E.M., n=4 in PBS, n=3 in cMpl agonist, n=2 in aged mouse group.

Analysis of BM HSPC compartments demonstrated little difference in progenitor cells (CLPs, CMPs, GMPs and MEPs) between different groups (Figure 15A,B), and significant increase (Figure 15C) in HSC containing fractions (LKS $CD34^-$, LKS $CD34^-CD48^-CD150^+$ =LKS $CD34^-$SLAM, LKS $CD34^-CD48^-CD150^{low}EPCR^{low/neg}$ and LKS $CD34^-CD48^-CD150^{high}EPCR^{high}$) in aged mice compared to PBS treated mice, as well as in cMpl agonist treated mice compared to PBS treated mice (Figure 15). Remarkably cMpl agonist treatment lead to more pronounced increase (>10 fold change cMpl agonist vs PBS treated groups) in phenotypic HSCs compared to natural ageing (4-5 fold change aged vs PBS treated).

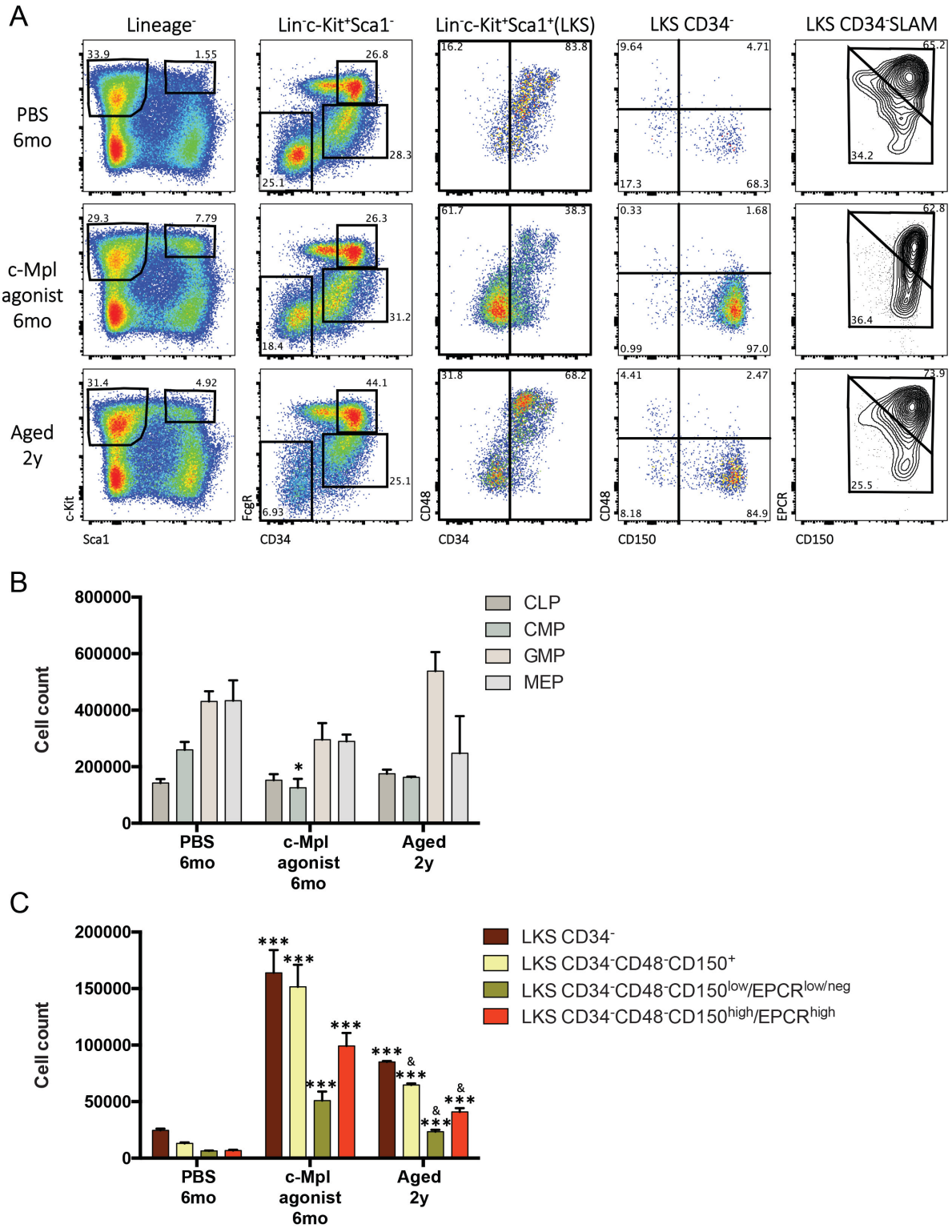

Figure 15: **Phenotypic increase in HSCs upon 6month of cMpl agonist treatment. A.** Representative FACS plots and gating strategy for HSPC populations; B. Absolute numbers of progenitor cells: CLP ($Lin^-IL7R^+cKit^+Sca1^+$), CMP ($Lin^-cKit^+Sca^-CD34^+FcgR^{low}$), GMP ($Lin^-cKit^+Sca1^-CD34^+FcgR^{high}$), MEP ($Lin^-cKit^+Sca1^-CD34^-FcgR^{low/neg}$) data represent mean value ± S.E.M.; C. Absolute numbers of HSPCs: LKS $CD34^-$, LKS $CD34^-CD48^-CD150^+$, LKS $CD34^-CD48^-CD150^{low}EPCR^{low/neg}$, LKS $CD34^-CD48^-CD150^{high}EPCR^{high}$ data represent mean value ± S.E.M, n=2-4.

The observation that there is significant and persistent increase in HSCs upon cMpl agonist stimulation raises the question, whether cMpl agonist can modulate stroma cell populations that support the function of expanded cells. Therefore, I also analyzed the stroma cell compartment in long bones of mice treated for 6 month with PBS or cMpl agonist and compared it to aged animals (Figure 16).

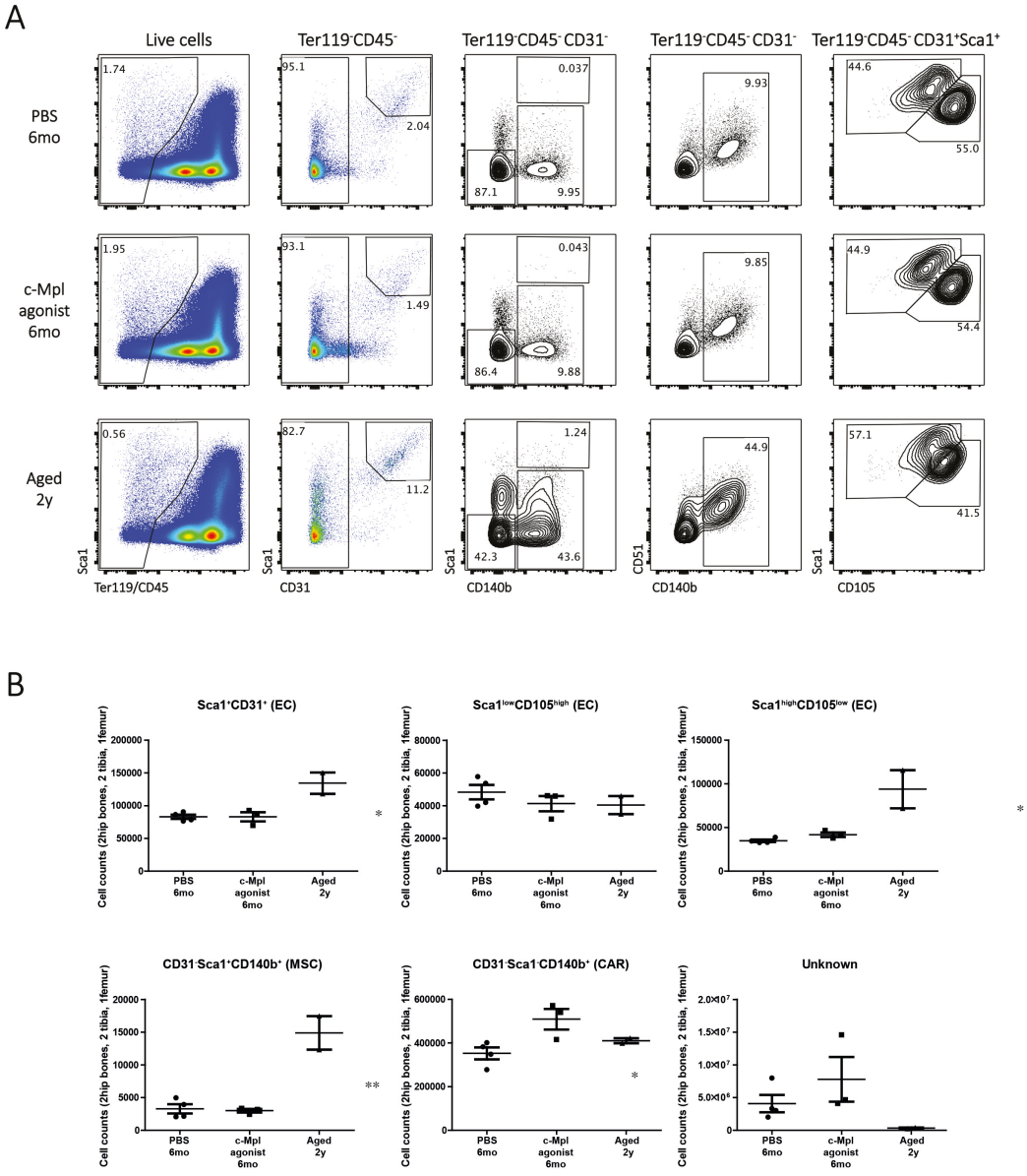

Figure 16: **Phenotypic stroma cell compartment upon cMpl agonist treatment. A.** Representative FACS plots and gating strategy for stroma cell gating; B. Absolute numbers of stroma cell populations: EC ($Ter119^-CD45^-Sca1^+CD31^+$), Sinusoidal endothelial cells ($Ter119^-CD45^-CD31^+Sca1^{low}CD105^{high}$), Arterial endothelial cells ($Ter119^-CD45^-CD31^+Sca1^{high}CD105^{low}$), MSC ($Ter119^-CD45^-CD31^-Sca1^+CD140b^+$), CAR ($Ter119^-CD45^-CD31^-Sca1^-CD140b^+$) and unknown population ($Ter119^-CD45^-CD31^-Sca1^-CD140b^-$) data represent mean value ± S.E.M, n=4 in PBS, n=3 in cMpl agonist, n=2 in aged mouse group, data was analyzed with paired, 2- tailed Student T-test, * $p<0.05$, ** $p<0.01$.

The analysis of the non-hematopoietic stromal cell compartment revealed that upon cMpl agonist treatment a stromal subpopulation defined by the marker combination $Ter119^-CD45^-CD31^-Sca1^-CD140b^+$ that contains CAR cells is increased ($p<0.05$), as compared to respective populations in PBS treated animals. In contrast to this, different subpopulations of stroma cells are increased in aged mice (in comparison with PBS treated animals) - endothelial cells ($Ter119^-CD45^-Sca1^+CD31^+$), specifically arterial endothelial cells ($Ter119^-CD45^-CD31^+Sca1^{high}CD105^{low}$) and MSC ($Ter119^-CD45^-CD31^-Sca1^+CD140b^+$). Notably, these are all subpopulations that are characterized by Sca1 expression, which might be indicative of low-grade inflammation in aged mice (Figure 16).

Overall, the phenotypic increase of HSCs that is observed in both natural ageing and long-term cMpl agonist treatment shows a direct correlation with increase in non-hematopoietic cell populations: in ageing endothelial cells, specifically Sca1 high expressing arteriolar cells and MSCs, are increased, while in cMpl agonist stimulation CAR cells are increased. These findings will be further investigated in collaboration with Cesar Nombela-Arrieta.

Extrinsic factors that regulate HSC ageing phenotype

To dissect ageing-associated extrinsic factors, I performed antibody-based protein arrays with young versus aged cell lysates from total BM (Figure 17). I found 39 candidate factors up-regulated in aged BM. These include adhesion molecules, pro-inflammatory cytokines, chemokines, and growth factors (Figure 17). To further dissect systemically or locally produced ageing-associated factors, a transcriptomic analysis of total BM cells (including stroma cells and hematopoietic cells) was performed. Four proteins were identified, IL-1α, IL-1β, MIP-2 and RANTES (Ccl5), all of which are known to be associated with inflammation. Ccl5 was excluded from the analysis as it had been already reported to cause myeloid skewing of HSCs during ageing (Ergen et al. 2012).

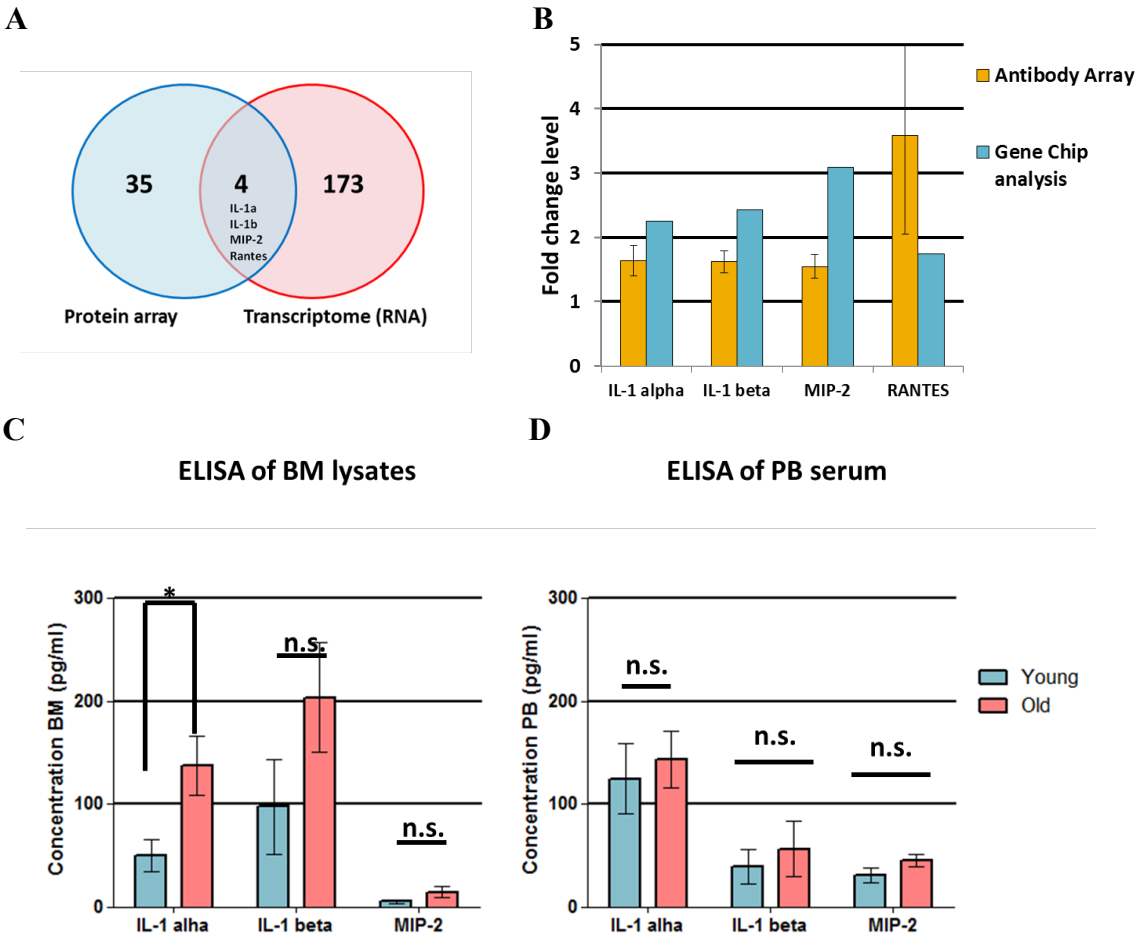

Figure 17: **Extrinsic factor screening. A.** Intersection of two approaches proteomics and transcriptomics **B.** Fold change (Young vs old) for candidates in intersection. C-D. Validation of intersection candidates BM lysates (C, left) or PB serum (D, right) were measured by ELISA. Statistical analysis was done by Student T-test, P value is indicated above the graph.

Other candidates were validated by ELISA to measure the concentration of these proteins in BM lysate and PB serum. IL-1α and IL-1β were up-regulated in aged BM, but not in serum, suggesting its local production in BM that differs upon ageing (Figure 17). MIP-2 showed no significant difference between young and aged in both BM and PB serum.

Therefore, I further focused on IL-1α, and IL-1β, and aimed to investigate the effect of IL-1α and IL-1β on young HSC cell division, and dissect whether there is any difference in the action between IL-1α and IL-1β. Both IL-1α and IL-1β belong to IL-1 family and use same receptor, IL-1RI and IL-1RII and the downstream signalling, however, their action on HSC divisional dynamics might be different (Garlanda et al. 2013). To address these questions, I employed the CFSE-dilution assay. CFSE-labelled young (8-12 week old) LKS was transferred into non-irradiated steady-state young adult recipients (Figure 18). Similar to experimental scheme in Figure 7, one week after transplantation mice were injected with PBS or IL-1α and IL-1β (1mg per mouse) for 5 consecutive days. Three weeks after the transfer, phenotypic BM analysis demonstrated that upon IL-1α treatment most of donor LKS had undergone at least 2 divisions, as compared to PBS control. Treatment with IL-1β has tendency of activating HSCs to proliferate. In both cases, HSCs seem to differentiate rather than self-renew, as cells that had been induced to divide upon IL-1α or IL-1β administration do not express stem and progenitor cell markers cKit, Sca-1 as PBS control (Figure 18).

The next question to ask was whether young and aged HSCs in the same context respond differently to proliferative signals through IL-1 receptor ligation. CFSE-labeled young adult (8-12 week old) or aged (>2years old) LKS was transferred into non-irradiated steady-state young adult recipients. One week after transplantation mice were injected with PBS or IL-1α (1mg per mouse) daily for 5 days (Figure 19). Three weeks after transfer, phenotypic BM analysis demonstrated that upon IL-1α treatment most of young donor LKS had undergone at least 2 divisions, as compared to PBS treated animals where some of aged LKS remained undivided (Figure 19).

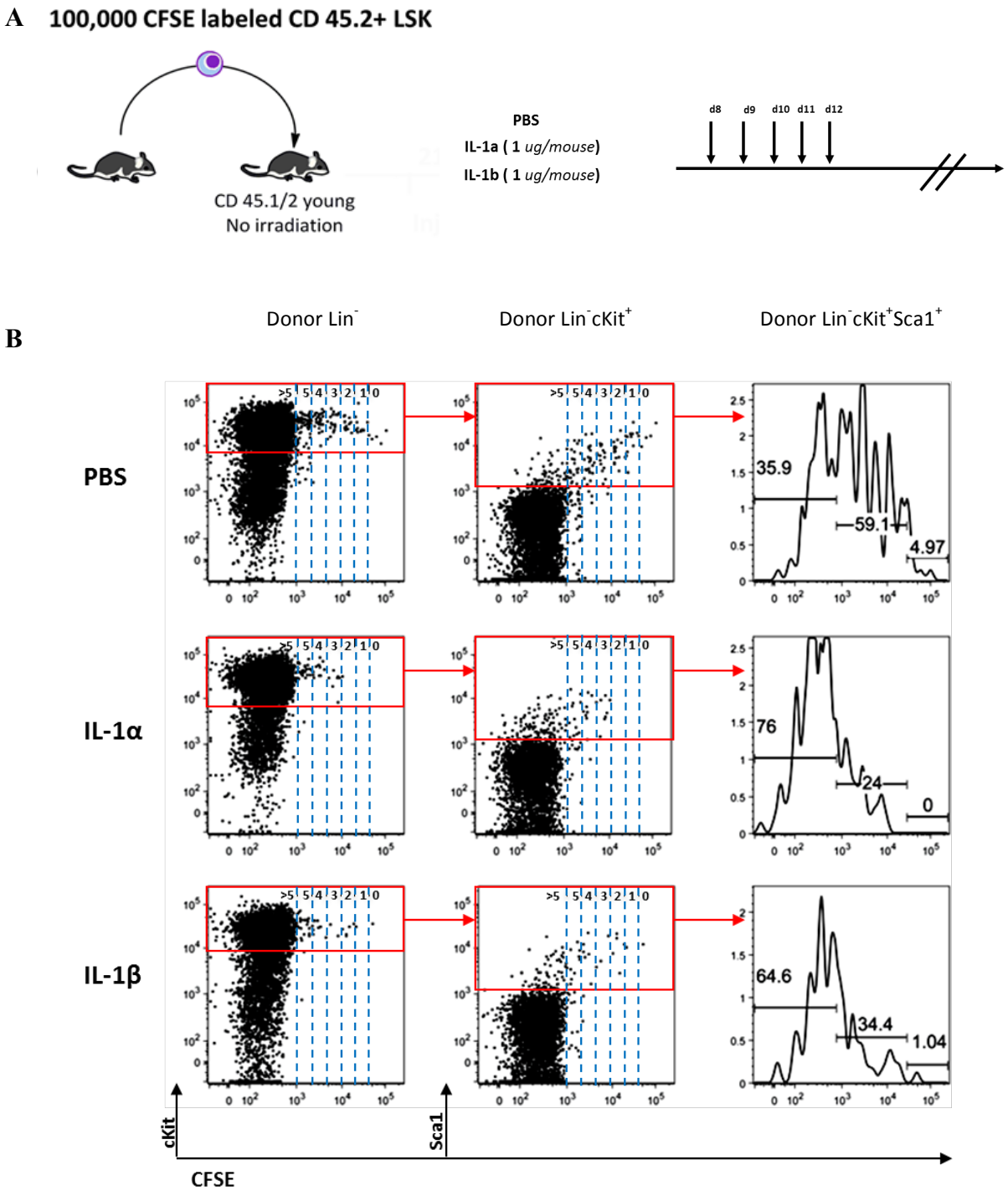

Figure 18: **Impact of IL-1α and IL-1β signalling on LKS cell division and differentiation. A.** Experimental design. **B.** Representative dot plot analysis of lineage-depleted BM cells gated on donor Lin$^-$ cells from animals treated with PBS, IL-1α or IL-1β (1mg per mouse, x5 injections). Dashed lines indicate each division.

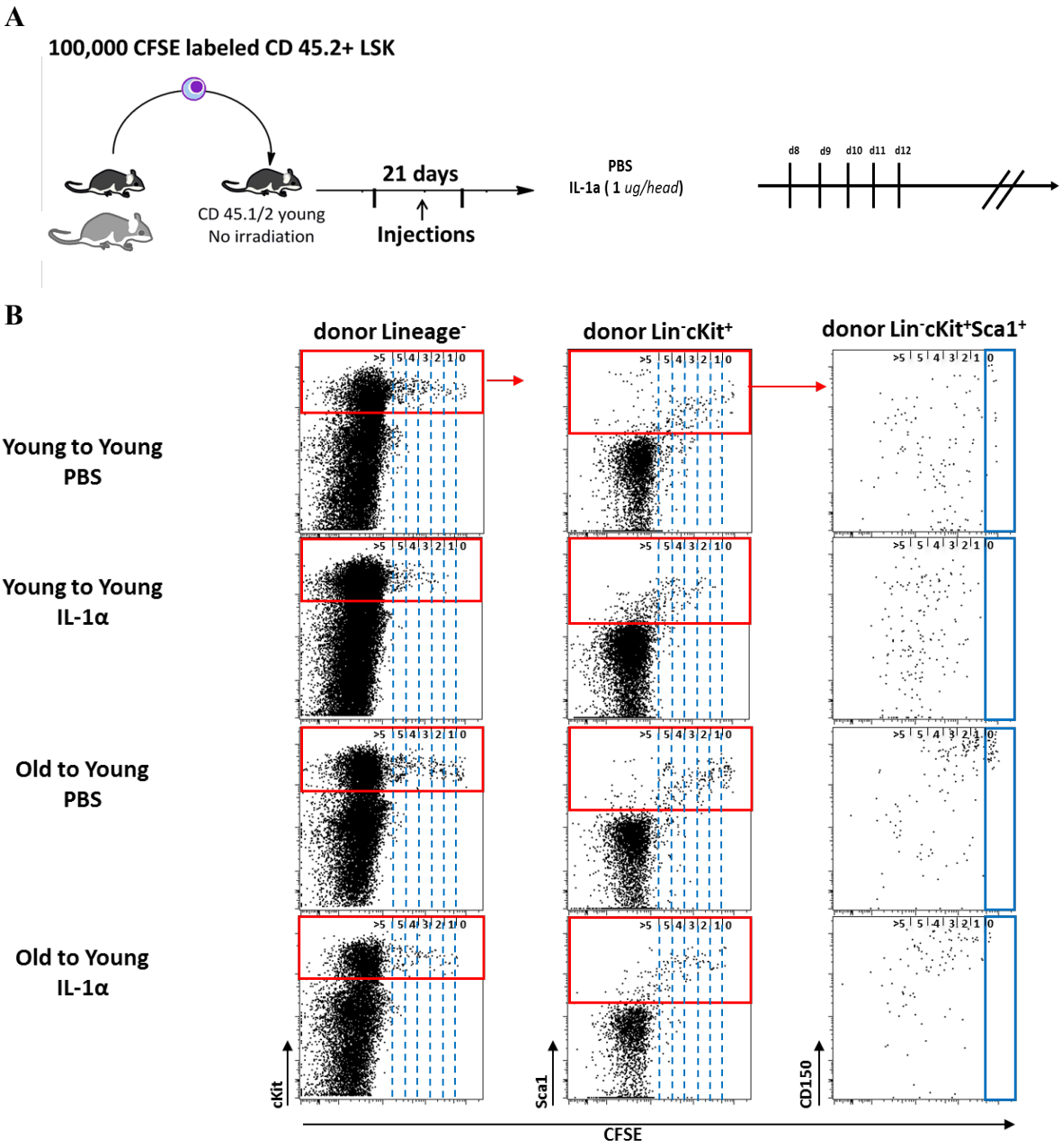

Figure 19. Impact of IL-1α treatment on young and aged HSC cycling activity. A. Experimental design. **B.** Representative dot plot analysis of lineage-depleted BM cells gated on donor Lin$^-$ cells from animals treated with PBS, IL-1α (1mg per mouse, x5 injections). Dashed lines indicate each division.

We further investigated the role of IL-1 signaling in context of ageing of the hematopoietic system by analyzing HSPC and mature blood cell compartments in young and old WT and IL-1RI KO mice. Highly purified HSC ($Lin^-cKit^+Sca1^+CD150^+CD34^-$) were significantly increased with age in WT animals ($p<0.01$), and to lesser extent in IL1RI KO mice ($p<0.01$) (Figure 20). PB analysis demonstrated that cell lineage composition in PB of aged WT animals was significantly ($p<0.05$) skewed towards myeloid lineage as compared to that of young WT mice (old WT PB, myeloid mean% = 70.84 vs young WT PB, myeloid mean%=46.69) (Figure 20). Although this myeloid skewing occurred in PB of aged IL1RI KO mice (old IL1RI KO PB, myeloid mean% = 47.04 vs young IL1RI KO PB, myeloid mean% = 30.38), the cell lineage composition in old IL1RI KO PB was less biased than in old WT PB, and similar to young WT PB. These results indicate a potential role of IL-1 signaling in mouse HSC aging, although the aging phenotype is not completely abrogated in aged IL1RI KO mice. This might indicate synergic effects of several other factors in HSC aging, as e.g. Rantes and IGF-1 (Ergen et al. 2012; Mayack et al. 2010; Nygren et al. 2006).

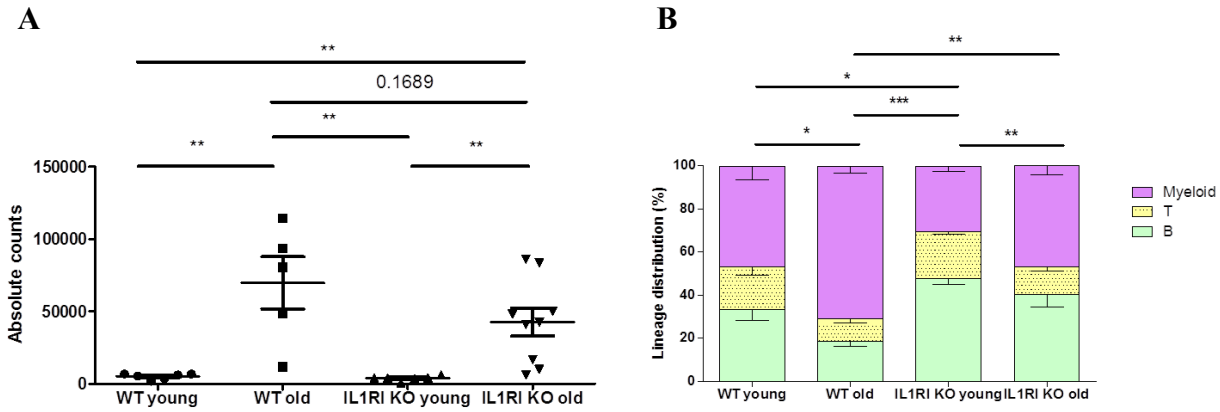

Figure 20. Immunophenotype analysis of young and aged WT and IL1RI KO mice. A. Absolute numbers of BM $Lin^-cKit^+Sca1^+CD150^+CD34^-$ cells, data represent individual variation, mean and S.E.M.; B. FACS analysis of blood composition, relative % of total $CD45^+$ cells: violet color represents myeloid lineage ($CD45^+CD3^-CD19^-$), yellow color Tcells ($CD45^+CD3^+CD19^-$), green Bcells ($CD45^+CD3^-CD19^+$), graph shows mean and S.E.M. *p<0.05, **p<0.01, ***p<0.001 Data is summary of three independent experiments, n=5-9.

SIGNIFICANCE

Understanding of the mechanisms underlying the natural ageing process will provide a basis for regenerative medicine approaches. If the findings for mouse HSC ageing will be confirmed for human HSCs, strategies for rejuvenation therapies could be identified which will improve the quality of life in the elderly population and possibly reduce the risk of acquiring hematopoietic neoplasia. And finally, similar mechanisms as found in HSC ageing could hold true in other somatic stem cell organ systems.

CONTRIBUTIONS

In addition to what is going to be published and is planned to be published (listed above in sections research article 1 and 2), I have contributed to various publications, book chapter and review:

a) Research article (*in preparation*)

Direct TLR4-TRIF-ROS-p38 signalling in hematopoietic stem cells induces proliferation and decreases competitive fitness

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b) Research article (*submitted*)

Chronic interleukin-1 drives haematopoietic stem cells towards precocious myeloid differentiation at the expense of self-renewal

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c) Research article (*Published:* J Immunol. 2014 Nov 15;193(10):5273-83)

Microbiota-derived compounds drive steady-state granulopoiesis via MyD88/TICAM signaling.

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Fas (CD95) expression in myeloid cells promotes obesity-induced muscle insulin resistance

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Mouse Genetic Background and Human Hematopoietic Stem Cells Biology; Tips for Humanization

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f) Review article (*in preparation*)

Inflamm-Ageing of Hematopoiesis and Hematopoietic Stem Cells

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DISCUSSION AND OUTLOOK

Hematopoietic blood cell production is dependent on a rare BM cell population – HSCs. While the hematopoietic progenitor system as whole is highly proliferative, HSCs as a population remain mostly quiescent. It was previously demonstrated that various stress conditions can activate HSCs towards proliferation (Takizawa et al. 2011; Takizawa & Manz 2011; Baldridge et al. 2010; Essers et al. 2009; Beerman et al. 2013).

The HSC-mobilizing agent G-CSF was reported to activate HSCs towards proliferation, as defined by Pyronin Y staining (Morrison et al. 1997) and BrdU uptake (Wright, Cheshier, et al. 2001). However, in these studies G-CSF was administered after Cyclophosmomide (CY) treatment, an alkylating agent, which induces cross-linkage of DNA strands. This result thus has to be interpreted with caution, as CY treatment eliminates cycling cells and presumably might cause an increase in inflammatory cytokines and thereby might induce remaining dormant HSCs to cycle. G-CSF as a single substance was also suggested to induce cell cycling, defined by BrdU retention or cell cycle snapshot analysis (Wilson et al. 2008; Walter et al. 2015). As proliferative agent, G-CSF was also proposed to be used in combination with chemotherapy to eradicate leukemia, because cycling leukemia initiating cells (LIC) should be more sensitive to chemotherapeutic agents (Saito et al. 2010). In contrast, I found with more sensitive divisional tracking than BrdU retention (Takizawa et al. 2011) that G-CSF alone, even at a higher dose than that used in previous studies, does not drive dormant HSCs into proliferation as defined by HSC function. This result may explain why G-CSF administration in clinical AML trials in combination with chemotherapy neither led to better responses, nor induce long lasting aplasia due to HSC eradication (Estey et al. 1994; Ohno et al. 1994).

The data on the role of thrombopoietin receptor signaling on HSC cell cycling activity is controversial. Some studies indicate that THPO-cMPL signaling supports quiescent HSCs, defined by the fact that blocking of this signaling leads to loss of self-renewal of HSCs (Yoshihara et al. 2007; Qian et al. 2007). Mice treated with blocking antibody and 5FU allow high levels of donor cell engraftment (Yoshihara et al.). Another study (Qian et al.) indicated that $THPO^{-/-}$ adult mice have 150 fold less HSCs and $THPO^{-/-}$ HSCs have rapid cell cycling activity. Both studies from Yoshihara et al. and Qian et al. demonstrated that the cell cycle inhibitor p57 is downregulated in context of non-functional cMPL/THPO signaling, i.e. in situations where signaling is blocked by AMM2 antibodies in LKS cells, or in $THPO^{-/-}$ LKS $Flt3^{-}$

cells, which both results in increased cell cycle entry, and thereby gradual loss of self-renewal capacity of HSCs. In contrast enhanced THPO stimulation increases p57 expression and LKS cells increase their quiescence as defined by side population (tip to basal SP ratio) analysis. However higher doses (200µg/kg (Walter et al. 2015), 500µg/kg and 1000µg/kg (Yoshihara et al. 2007)) of THPO again induced proliferation of LKS $CD34^{-/low}$ cells, as defined by snap-shot cell cycle analysis. Indeed TPO-signaling is also critical for efficient hematopoietic engraftment and reconstitution after lethal irradiation transplantation, as mice transplanted with $THPO^{-/-}$ cells had a 50% survival after 125 days, and this defect can be compensated by transplantation with a 4-fold higher dose of total bone marrow cells from THPO-/- mice (Fox et al. 2002). Moreover, THPO administration can also reinforce the reconstituting potential of $THPO^{-/-}$ cells (Fox et al. 2002). Using the proliferative history of the cells, I unambiguously demonstrated that stimulation of cMPL/THPO signaling by the cMpl-agonist Nplate continuously (with doses of 100µg/kg, 10µg/kg and 1µg/kg) can induce uniform proliferation of dormant HSCs (leaving no cells in non-divisional gate or single cell in case of 1µg/kg). Most importantly, the self-renewal of proliferated HSCs was maintained. These results were confirmed when mice were first treated with cytokines, followed by treatment with 5FU, an anti-proliferative agent which specifically eradicates only proliferating cells. This also indicates a potential clinical application of cMpl-agonists. The proliferative property of the drug might be used in clinical approaches as pre-treatment in combination with myeloablative therapies, followed by allogeneic HSCT. This might help to eradicate malignant HSCs/LICs more efficiently (assuming that human healthy HSCs and LICs are as well sensitive to cMpl agonists) and also might allow to reduce the dosage of myeloablative therapy to create space for incoming HSCs from HSCT. Currently I am investigating whether cMpl-agonist can indeed activate human healthy HSCs into cell cycle. If cMpl-activation can activate pre-malignant HSCs/LICs remains to be determined.

Ageing of the hemato-lymphoid system is characterized by declined function and replenishment of acquired and humoral immunity, increased dependency of host defense on innate immune barrier against pathogens, and a reduced regenerative capacity of early hematopoietic progenitor cells, associated with increased incidence of infection, hematopoietic malignancies and dysplasia (Warren & D. J. Rossi 2009). Some of these differences are attributed to aged HSC, which reduce their BM-homing efficiency, self-

renewal capacity, and have myeloid-biased differentiation. The HSC ageing phenotype can be experimentally recapitulated by driving HSCs to proliferation, as it was previously demonstrated by serial transplantation, or multiple 5FU administrations (Dykstra et al. 2011; Beerman et al. 2013). To possibly preserve HSC function or even reverse ageing-associated alterations, it is crucial to understand what factors or what mechanisms are controlling HSC turnover and hematopoietic ageing. Recently our group demonstrated (Takizawa et al. 2011) that at any given time the HSC pool consist of active cycling and quiescent fractions. These two pools can reversibly switch their status over time, i.e. quiescent HSCs can be activated upon demand (systemic infection such as acute LPS treatment) and HSC with extensive proliferative history and switch back to dormancy and tend to be dormant, indicating an intrinsic and extrinsic control of c HSCs activity.

My results indicate that aged HSCs are more quiescent than young HSCs in an isochronic aged environment, and they are induced to proliferate in a heterochronic young environment. When I tested the function of quiescent HSCs, young HSCs exhibited lymphoid biased output and aged or serially transplanted HSCs demonstrated myeloid biased output. This did not change in young or aged environments. While cycling aged HSCs that had been exposed to an isochronic aged environment exhibit myeloid biased differentiation, cycling aged or serially transplanted HSCs that had been exposed to a heterochronic young environment switch, surprisingly, towards lymphoid biased repopulation. This indicates a possibility to reverse ageing-associated myeloid biased lineage output of HSCs by exposing them to young environmental factors.

Our results also indicate that long-term application of cMpl agonist leads to phenotypic HSC expansion and might also exhaust the HSCs. Indeed, after cMpl-agonist treatment, peripheral blood composition changes towards a higher ratio of myeloid lineage, and HSCs exposed to long term cMpl-administration tend to increase in number and also increase their quiescence, recapitulating partially a phenotype of HSC ageing. In future studies I will investigate whether the functionality of HSCs is affected by long-term treatment on a per cell level, similar to what is observed in natural ageing. The phenotypic increase in aged HSCs and in chronic cMpl-agonist treated mice is followed by an increase in different non-hematopoietic stroma cell populations: aged mice have higher numbers of MSCs, and EC-containing cell fractions, while cMpl-agonist treatment lead to increase in a CAR cell containing fraction. This needs to be further investigated and confirmed on reporter

mouse models and by 3D imaging techniques (e.g. CXCL12-GFP mice that would allow more faithful discrimination of CAR cells), both currently ongoing in collaboration with Cesar Nombela-Arrieta.

To evaluate cell-extrinsic factors involved in HSC ageing, I examined the molecular composition of young and aged BM lysates by AB based arrays. I found that aged BM compared to young BM is composed of a differential cytokine milieu, the majority of which were inflammatory cytokines. Comparative data of gene expression and protein expression revealed IL-1α, IL-1β as elevated in aged BM (indicating likely local production), and both were higher compared to young BM lysates. However, neither IL-1α nor IL-1β were elevated in serum of aged animals, supporting our assumption from proteomics and transcriptomics analysis that these were mostly locally produced.

I then tested effects of IL-1α and IL-1β on HSC division rates. IL-1α induced HSC proliferation, whereas IL-1β had a less pronounced proliferative effect. I thus did not further focus on IL-1β in my ageing-associated study, however, I picked it up again in a collaboration with E. Pietras and E. Passegue, where the focus of research was the dissection of the role of IL-1 signaling in young adult HSCs and its influence on myeloid differentiation. In my studies on HSC ageing, I analyzed whether aged and young HSCs respond similarly to proliferative signals transmitted by IL-1α. I found that aged HSCs proliferate slower than young HSCs in response to IL-1α. This indicates the possibility that aged HSCs are less fit to respond to proliferative signaling, either due to a higher endogenous drive to quiescence based on proliferative history, or due to downregulated IL-1R signaling or both.

Next, I attempted to dissect the role of IL-1 signaling in ageing, by analyzing aged versus young IL1RI KO and WT mice. Results indicated that aged IL1RI KO mice have fewer numbers of phenotypic HSCs in BM than aged WT mice. This might explain the observed different mature blood cell composition: while aged WT mice hematopoiesis is myeloid biased, aged IL1RI KO mice have balanced lineage ratios. This will be further evaluated in detail, by analyzing HSC compartments upon inhibition of IL-1 signaling in vivo. It still remains to be determined what cell population is the main provider of IL-1 in the bone marrow of aged mice. Possible cell types are both of hematopoietic origin (macrophages), and non-hematopoietic origin. The latter might include endothelial cells (as they were reported to express IL-1α in other organ systems (Garlanda et al. 2013)) and mesenchymal stroma cells. In addition, it will be interesting to determine whether HSCs with myeloid-

biased potential and HSCs with lymphoid-biased potential have different levels of IL-1 receptor expression.

Given the above described data and prior literature, IL-1 inhibition might represent a promising target for systemic rejuvenation as it seems to be involved in different organ ageing processes (Fisher et al. 2002).

CONCLUSIONS

1. The thrombopoietin mimetic cMPL -agonist drives dormant mouse HSCs towards proliferation, while maintaining their self-renewal capacity.
2. G-CSF has little impact on dormant HSCs proliferation.
3. Long term cMpl-agonist stimulation leads to phenotypic HSC pool expansion and ultimately increases their quiescence.
4. Extensive proliferative history imprints an intrinsic program for quiescence and myeloid-biased differentiation on HSCs.
5. The function of aged HSCs can be modulated via environmental cues.
6. IL-1 signaling can contribute to HSC aging and is important for HSC to myeloid cell differentiation. Constitutive knock out of IL-1 reduces myeloid over lymphoid lineage dominance.

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